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NON-ENERGY DEPENDENT NADPH PRODUCTION FOR  
STEROID HYDROXYLATION IN  
RAT ADRENAL MITOCHONDRIA



ROBERT PARK

1971

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NON-ENERGY DEPENDENT NADPH PRODUCTION  
FOR STEROID HYDROXYLATION IN RAT ADRENAL MITOCHONDRIA

by

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## INTRODUCTION

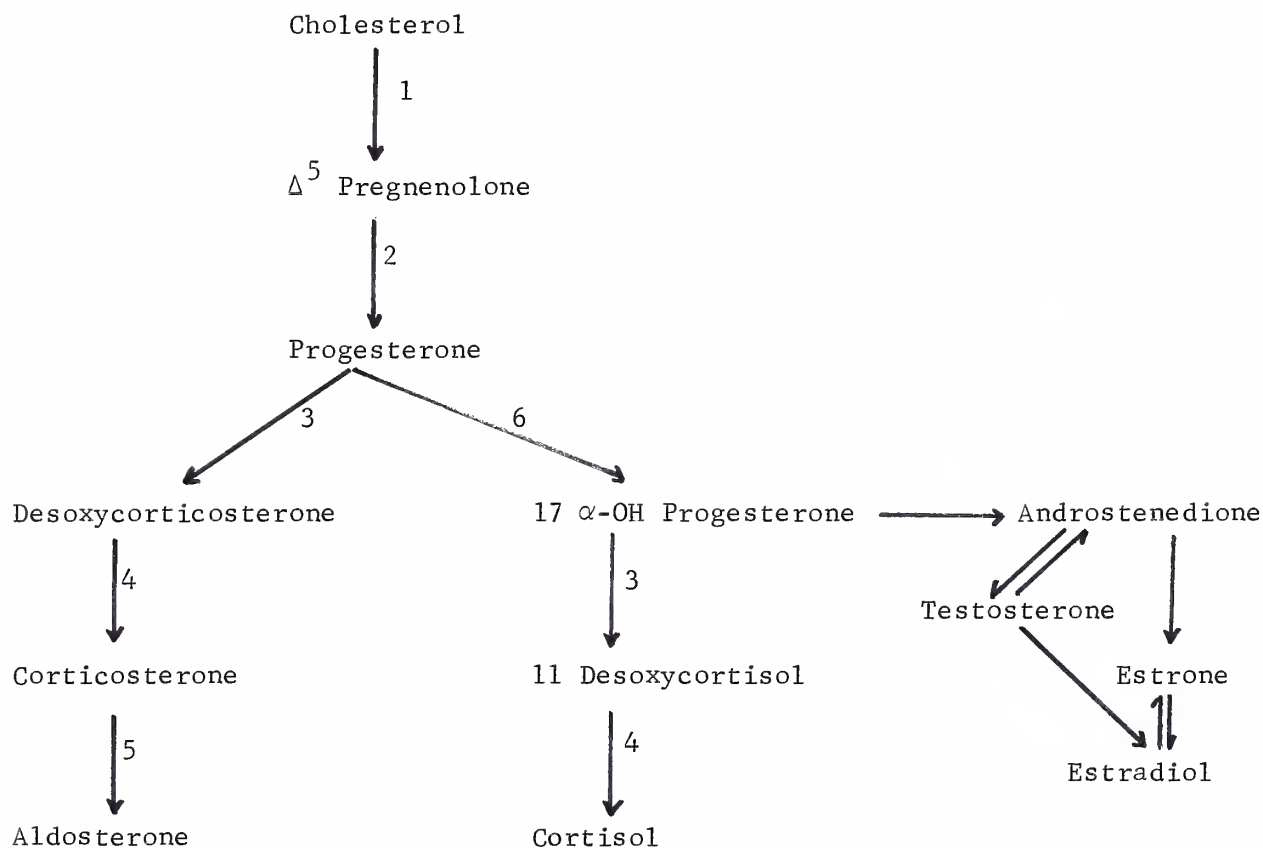
Certain of the biochemical reactions in adrenal steroid biosynthesis consist of hydroxylations of cholesterol to produce the biologically active steroids corticosterone, aldosterone, and cortisol. These reactions occur in the mitochondria, endoplasmic reticulum, and soluble fractions of the adrenal cell(Fig. 1).

The initial reaction is the side chain cleavage of cholesterol to pregnenolone(1-5). This reaction occurs within the mitochondria. The pregnenolone must diffuse out of the mitochondria for the next reaction is catalyzed by the NAD-linked  $3\beta$ -ol dehydrogenase and  $\Delta^5$  ketoisomerase. This reaction converts pregnenolone to progesterone(6,7) and takes place in the soluble-microsomal fraction. At this point, the biosynthetic scheme diverges into two major pathways. In the one terminating with cortisol, a 17 hydroxylase in the microsomal fraction forms  $17\alpha$ -OH progesterone, which is then converted to 11 desoxycortisol(compound S) by a microsomal 21 hydroxylase(6-9). For the final reaction, compound S enters the mitochondria again where  $11\beta$  hydroxylase forms cortisol(6,10), the major glucocorticoid in man.

The second major pathway from progesterone starts with the formation



Figure 1. Biosynthetic Scheme in the Formation of Adrenal Corticosteroids from Cholesterol



1. Side chain cleavage enzyme in mitochondria
2. 3  $\beta$ -ol dehydrogenase and  $\Delta^5$  ketoisomerase in soluble-microsomes
3. 21 hydroxylase in microsomes
4. 11 $\beta$  hydroxylase in mitochondria
5. 18 hydroxylase in mitochondria
6. 17 hydroxylase in microsomes



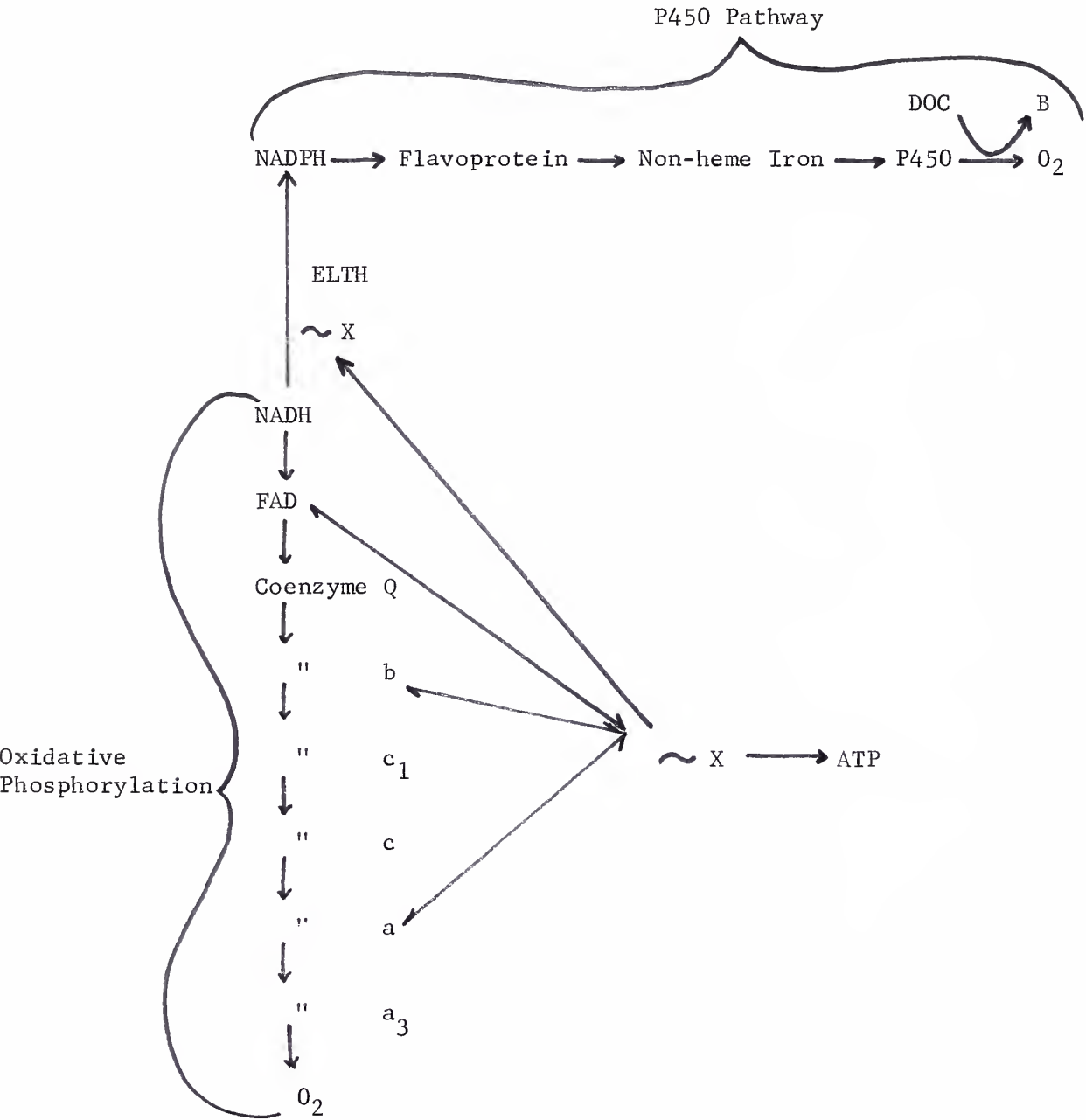
of desoxycorticosterone(DOC) by the microsomal 21 hydroxylase. DOC enters the mitochondria for the last two reactions. The  $11\beta$  hydroxylase(11) converts DOC to corticosterone(the major glucocorticoid in the rat). Finally, the 18 hydroxylase(12) converts corticosterone to aldosterone, the principal mineralocorticoid. All of the hydroxylase reactions are catalyzed by mixed function oxidases(see below) which require NADPH and molecular oxygen. In summary, three mitochondrial NADPH-dependent reactions are involved in corticosteroidogenesis: the side chain cleavage,  $11\beta$  hydroxylation, and C-18 hydroxylation. The other biosynthetic steps take place in the soluble cell fraction and endoplasmic reticulum.

Adrenal cortex mitochondria are of especial interest because they contain two electron transport chains. In addition to the pathway associated with oxidative phosphorylation present in nearly all mitochondria, adrenal cortex mitochondria contain a Cytochrome P450 pathway which catalyzes mixed function oxidase reactions(13). The components of this pathway include NADPH(14), a flavoprotein(15,16), a non-heme iron containing protein called adrenodoxin(17), and Cytochrome P450(15,16,18). Molecular oxygen acts as the final electron acceptor(Fig. 2). Recently, various workers have found that the activity of the P450 pathway is associated with mitochondrial steroid hydroxylation, i.e., the cholesterol side chain cleavage and the  $11\beta$  and C-18 hydroxylations. The stoichiometric relationship between the P450 pathway and steroid biosynthesis is such that 1 mole of steroid is hydroxylated for each mole of NADPH and oxygen consumed.

Even before the significance of the P450 pathway was known, the



Figure 2. Relationship Between the Classic Electron Transport and P450 Pathways in Rat Adrenal Mitochondria.





important requirement of NADPH in the steroid biosynthetic reactions was demonstrated(14,20,23). Workers postulated that the mechanism of action of adrenocorticotrophic hormone(ACTH) was via pathways causing an increase in the concentration of NADPH. Haynes and Berthet(22) and subsequently McKerns(21) suggested that ACTH activated adenyl cyclase and the cyclic 3'5' adenosine monophosphate(cAMP) formed enhanced phosphorylase activity. Increased amounts of glucose-6-PO<sub>4</sub> were metabolized via the hexose monophosphate shunt(HMS) wherein glucose-6-PO<sub>4</sub> dehydrogenase(G6PD) and other NADP-linked enzymes generated increased levels of NADPH. Other workers(24,25) found that ACTH caused only a direct increase in activity of G6PD. In either case, an increase in NADPH was thought to result, thus leading to enhancement of corticosteroidogenesis.

Although it was known that G6PD and the HMS were localized in the extramitochondrial soluble cell fraction several of the hydroxylations are mitochondrial(28). None of the workers postulated mechanisms whereby NADPH produced outside the mitochondria could gain access to the intramitochondrial space. It had long been known that the intact mitochondrial membrane is essentially impermeable to the pyridine nucleotides(26,27), yet McKerns felt that the HMS pathway played a central role in providing NADPH for steroid biosynthesis(28). Brownie and Grant (11) had shown that certain Krebs cycle intermediates such as isocitrate,  $\alpha$ -ketoglutarate, and malate could support mitochondrial 11 $\beta$  hydroxylation. Presumably these substrates penetrate the mitochondrial membrane and are metabolized to yield NADPH.

The demonstration of the P450 pathway led to the elucidation of its relationship to the classic electron transport chain and oxidative



phosphorylation. It has been shown that the two electron transport pathways are connected by a transhydrogenase which reduces NADP at the expense of NADH. The reaction is enhanced in the presence of ATP or high energy intermediates of oxidative phosphorylation(19). (See Fig. 2) Because this pyridine nucleotide transhydrogenase is dependent on energy, it is called the energy linked transhydrogenase(ELTH). Ernster and Lee(29) proposed the following explanation of the reaction:



where  $\sim\text{X}$  represent a non-phosphorylated high energy intermediate. In the absence of  $\sim\text{X}$ , the reaction has an equilibrium constant(K) of 0.79 and therefore is almost freely reversible. However, in the presence of  $\sim\text{X}$  the equilibrium constant(  $K = \frac{(\text{NAD})(\text{NADPH})}{(\text{NADH})(\text{NADP})}$  ) may be as high as 480. In other words, energy drives the reaction far to the side of NADPH formation. In this manner, the ELTH can provide NADPH for steroid hydroxylation from intramitochondrial sources of NADH. In the absence of energy, this pathway is still functional but to a much lesser degree.

The ELTH thus provides one mechanism to explain the observations of Brownie and Grant(see above) and others that Krebs cycle intermediates metabolized by NAD-linked dehydrogenases can support steroid hydroxylation (30-32). In addition, work by Harding showed that uncouplers of oxidative phosphorylation inhibited succinate supported 11 $\beta$  hydroxylation (33). Likewise, Sauer demonstrated that steroid hydroxylation supported by  $\beta$ -OH butyrate and  $\alpha$ -ketoglutarate was strictly dependent upon the ELTH since KCN and rotenone, agents which inhibit the respiratory chain, completely abolished 11 $\beta$  hydroxylation(34). This work conclusively



demonstrates that the ELTH pathway is available only in the presence of energy. Furthermore, the significance of the ELTH is manifest in steroid hydroxylation supported by NAD-linked substrates, clearly demonstrating the functional dependence of steroidogenesis upon oxidative phosphorylation.

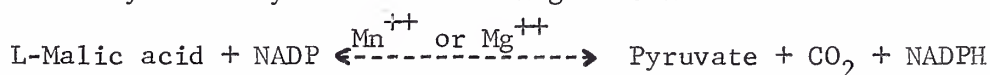
The pyridine nucleotide transhydrogenation is only one of several reactions which generate intramitochondrial NADPH for steroid hydroxylation. In addition, there are non-energy dependent mechanisms to make NADPH directly available for mitochondrial steroid hydroxylations. Such direct reactions could be operative in complete independence of oxidative phosphorylation, a situation which might be important when maximal rates of both steroid hydroxylation and ATP formation were required. These reactions are catalyzed by NADP-linked isocitric dehydrogenase (ICDH) and malic enzyme (ME).

The existence of a mitochondrial NADP-linked isocitric dehydrogenase was first demonstrated in liver tissue by Hogeboom and Schneider(35) and subsequently verified by Ernster and Navazio(36) and others(37). These workers were able to show that about 10% of the total NADP-linked ICDH activity was mitochondrial whereas the remainder was in the soluble fraction. Schneider and Hogeboom(38) demonstrated a similar distribution using human placental tissue. In the human adrenal, Studzinski and his colleagues gave evidence that only 3% of the NADP-linked ICDH was mitochondrial, and the rest was in the soluble fraction(24). In the rat adrenal Guerra et. al.(30) demonstrated NADP-linked ICDH activity; and Sauer showed that isocitrate supported  $11\beta$  hydroxylation is unaffected by respiratory



chain inhibitors(34) implicating high activity of the mitochondrial NADP-linked ICDH. In contrast, bovine adrenal mitochondria do not seem to utilize isocitrate as well as the rat; and in fact Goebell et.al.(39) have shown that over 90% of NADP-linked ICDH is extramitochondrial in bovine adrenals. Although isocitrate is known to be a good substrate for steroid hydroxylation, the available data show that differences exist regarding the contribution by the NAD and NADP linked enzymes in various animals(11,40). Thus, the rat adrenal seems to have a high activity of the NADP-linked ICDH, whereas bovine and human adrenal mitochondria rely more upon the NAD linked enzyme and the ELTH to support steroid hydroxylation within the mitochondria.

In addition to isocitrate, malic acid constitutes another Krebs cycle intermediate which can be metabolized by a mitochondrial NADP-linked enzyme. Malic enzyme(ME) was originally described by Ochoa(41), and Hsu and Lardy eventually isolated and characterized the enzyme from pigeon liver(42). Malic enzyme catalyzes the following reaction:



Malate is also metabolized by the mitochondrial NAD-linked malate dehydrogenase(MDH) to produce NADH and oxaloacetate.

Investigators have previously ascribed a role for ME in gluconeogenesis by suggesting that it provides oxaloacetate by converting pyruvate to malate which is then hydrogenated to oxaloacetate, a precursor of phosphoenolpyruvate(46-48). Others suggested that ME had a functional role in lipogenesis by providing sources of NADPH(43-45).

Studzinski et.al.(24) studied several NADP-linked dehydrogenases in the human adrenal and found that ME had such an extremely low activity as



to be barely measureable. Furthermore, the detectable ME activity was limited to the soluble fraction of the cell. In contrast, Simpson and Estabrook provided evidence in bovine adrenal mitochondria that ME was the major source of NADPH during malate and succinate supported steroidogenesis(49). With ME, as with NADP-linked ICDH, animal differences were noted. Using rats, Purvis proposed that ME was a minor source of NADPH in mitochondrial  $11\beta$  hydroxylation as opposed to bovine mitochondria(40). Furthermore, Peron and Tsang felt that ME was relatively unimportant in that malate was oxidized mainly via the NAD-linked malate dehydrogenase(50). Recently, however, Sauer was able to stimulate a malic enzyme-like activity\* in rat adrenal mitochondria by the addition of orthophosphate( $P_i$ ) or arsenate(As)(34). In the absence of  $P_i$  or As his findings were similar to those of Purvis in that the uncoupler-sensitive malate dehydrogenase was the major pathway for malate oxidation. However, upon the addition of  $P_i$ , there was an uncoupler-insensitive stimulation of steroid hydroxylation implying that malate was producing NADPH directly. Thus, in contrast to bovine mitochondria, rat adrenal mitochondria require the presence of  $P_i$  or As for malic enzyme activity to become manifest(49,51,52).

In summary then, rat, bovine, and perhaps human adrenal mitochondria show evidence that malate can be metabolized by non-energy dependent mechanisms in the production of intramitochondrial NADPH for steroid hydroxylation. The role of ME may therefore be similar to NADP-linked ICDH; together they constitute another mechanism (in addition to the

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\* Malic enzyme-like activity is defined as the production of pyruvate and NADPH from malate.



energy linked and non-energy linked transhydrogenases) whereby NADPH may be generated within the mitochondria.

Because ME has been shown to be present both within the mitochondria and in the soluble part of the adrenal cell, workers have postulated a shuttle mechanism to effect a net transfer of NADPH across the mitochondrial membrane using malate as the carrier molecule(53). The mechanism is essentially similar to that described originally by Boxer and Devlin(54) and more recently by Estabrook and Sacktor(55) in tissues other than the adrenal. Simpson and Estabrook have provided initial data that a malate shuttle may be operative in bovine adrenal cortex mitochondria(53). It has been suggested in rat adrenal mitochondria(56), but subsequently evidence was provided that it did not occur(50). As pointed out by other workers, the existence of a shuttle mechanism requires the presence of the enzyme both in the mitochondria and in the extra-mitochondrial soluble fraction. The enzyme must be capable of catalyzing both the forward and reverse reactions. In addition, the substrates which act as carriers for the pyridine nucleotides must be readily permeable to the mitochondrial membrane. In this manner, a cytosol malic enzyme would generate malate from pyruvate, CO<sub>2</sub>, and extramitochondrial NADPH. The mitochondrial ME could then oxidize malate back to pyruvate, CO<sub>2</sub>, and intramitochondrial NADPH--thus effecting the net transfer of reducing equivalents across the mitochondrial membrane. Such a mechanism could utilize the NADPH generated by the hexose monophosphate shunt and would substantiate McKerns' proposal(see p.3) that the HMS must play a central role in providing NADPH for steroid biosynthesis(28).



At present, the available data regarding non-energy dependent mechanisms for intramitochondrial accumulation of NADPH are incomplete, and different opinions exist concerning the contribution of NADP-linked ICDH and ME to adrenal corticosteroidogenesis. Furthermore, the relationship between these and other NADPH producing mechanisms needs clarification. The existence of several non-energy dependent enzymatic pathways may indeed imply that regulatory factors favor one pathway over another. The additional intriguing possibility of a malate shuttle has been proposed in bovine adrenals.

This project was therefore begun with the purpose of further exploring and clarifying the role of non-energy dependent mechanisms in intramitochondrial NADPH formation for adrenal steroid biosynthesis. Certain details of NADP-linked ICDH and ME have not been examined in the past, and their relative contribution to steroidogenesis is unclear. In addition, certain features of substrate entry and efflux from mitochondria were examined. Finally, because the possibility of an NADPH shuttle system has been raised, attempts were made to demonstrate the existence of both an isocitrate and malate shuttle. In summary, then, the emphasis in this thesis is placed on the non-energy dependent mechanisms operative in the intramitochondrial production of NADPH for steroid hydroxylations in the rat adrenal gland.



## MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 150-200 grams were used in these experiments. The rats were fed a normal laboratory chow ad libitum. In a typical experiment, 6-12 rats were killed by decapitation. The adrenals were quickly removed, chilled in ice cold 0.25 M Sucrose and adherent fat dissected away. The cleaned glands were then homogenized at 0-4° Cent. in 8 ml. of a medium containing 0.25 M Sucrose, 1 mM ethylenediamine-tetraacetate(EDTA), 30 mM TRIS-HCl, and 1% bovine serum albumin (pH 7.4) with 15 passes of a Teflon pestle in a 10 ml. glass homogenizer. Low speed centrifugation at 600g for 10 minutes removed nuclei, unbroken cells, and capsule fragments. The supernatant was decanted and stored on ice while the pellet was re-homogenized in 6 ml. of medium and centrifuged again. The combined supernatant solutions were centrifuged at 5200g for 20 minutes. The mitochondrial pellet was washed twice by resuspension in 3 ml. of medium and centrifuged at 5200g for 10 minutes. The final mitochondrial pellet was resuspended in 0.5-1.0 ml. of medium for use in the experiments. After correcting for protein content of the medium, the usual yield of mitochondrial protein was 0.5-1.0 milligram(mgm.) per pair of glands. Protein was determined by the Biuret method(57).



Sonication was carried out using a Model W185D Sonifier Cell Disruptor manufactured by Branson Sonic Power Company. Subcellular fractions were diluted 1:4 with 0.25 M Sucrose and sonicated at a power setting of 2-4 for 30 second periods per minute over 5 minutes at 0-4° Cent. Microsomes were isolated by centrifuging the supernatant from the 5200g-20 min. spin at 100,000g for 60 minutes. The microsomal supernatant was the soluble fraction.

Enzyme distribution experiments were done on sonicated subcellular fractions. NADP-linked ICDH activity was determined by spectrophotometric reduction of NADP in the presence of 8 mM d-isocitrate, 1 mM  $\text{MnCl}_2$ , 0.1 M TRIS-HCl pH 7.4, and 33 mM NADP using a Gilford Model 2000 Recording Spectrophotometer at 340 m $\mu$ . Malic enzyme activity was measured in the same manner using 10 mM malate, 10 mM  $\text{MgCl}_2$ , 0.1 M TRIS-HCl pH 7.4, and 20 mM NADP. The reaction was started by the addition of substrate. Blank cuvettes contained all additions except substrate.

Incubations and substrate efflux experiments were carried out in a medium containing 50 mM sucrose, 20 mM KCl, 1 mM EDTA, 30 mM TRIS-HCl pH 7.4, and 1% bovine serum albumin. When used, desoxycorticosterone and rotenone were added as ethanolic solutions. Substrate concentrations for d-isocitrate and l-malate were 10 mM. ~~K~~CN solutions were prepared immediately prior to use with 0.05 M TRIS-HCl pH 8.6.

In a typical substrate efflux experiment, 2 flasks containing 5-8 ml. of a medium with about 0.3 mgm. mitochondrial protein per ml. were incubated for 12-48 minutes at 37° Cent. Flasks were preincubated for several minutes, and the reaction started with the addition of mitochondria.



Orthophosphate was added at variable times during the incubation. 0.5 ml. aliquots for  $\alpha$ -ketoglutarate and pyruvate determinations were removed with a pipette at timed intervals and immediately centrifuged for 15 seconds in a Fisher Model 59 Semi-micro centrifuge. This would form a compact mitochondrial pellet from which the supernatant was decanted, iced, and assayed.  $\alpha$ -ketoglutarate was determined by spectrophotometric oxidation of NADPH at 340 m $\mu$  using glutamic dehydrogenase (Calbiochem). Pyruvate was determined in the same manner using lactic dehydrogenase (C. F. Boehringer).

Mitochondrial swelling experiments utilized 90 mM NH<sub>4</sub>-malate pH 7.6, and light scattering was read spectrophotometrically at 600 m $\mu$ . In the C<sup>14</sup>-malate uptake experiments, 50  $\mu$ Curies of C<sup>14</sup>-L-malic acid, specific activity 9.1 mCuries/mMole (New England Nuclear), was diluted so that the flask concentration of 10 mM malate had a theoretical activity of 317,500 counts per minute (CPM)/ml. of incubation medium. Tritiated water (New England Nuclear) had an activity of 133,000 CPM/ml. incubation medium. The mitochondria were centrifuged as in the substrate efflux experiments; the pellet was then lysed with 0.1 ml. of 7% perchloric acid and counted for radioactivity in a Model 2002 Packard Tricarb Scintillation Spectrometer; Bray's solution was used as counting medium.

Soluble fraction malic enzyme for the malate shuttle experiments was partially purified from pooled 5200g-20 min. supernatants which were then centrifuged at 100,000g for 60 minutes. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation of the soluble fraction was carried out at 0-4° Cent. The fractionated protein was centrifuged for 10 min. at 15,000g, then redissolved in 0.1 M TRIS-HCl pH 7.4. The fractions containing the most enzyme activity were pooled and



dialyzed for 24 hours against 0.03 M TRIS-HCl pH 7.4 with 2 mM mercaptoethanol at 0-4° Cent. with 3 exchanges of fluid. The enzyme was then concentrated in solid sucrose for 8-12 hours to reduce the volume and redialyzed for several hours to remove most of the sucrose. After re-assay for activity, the enzyme was used in the shuttle experiment. In addition to the incubation mix, the following reagents were included in the shuttle experiment: 300  $\mu$ M DOC, 10 mM Na-pyruvate, 50 mM NaHCO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, 1 mM Arsenite, 2 mM orthophosphate(P<sub>i</sub>), approximately 0.8 mgm. mitochondrial protein/ml. total incubation medium, and an NADPH generating system consisting of 0.5 mM NADPH, 10 mM glucose-6-PO<sub>4</sub>, and glucose-6-PO<sub>4</sub> dehydrogenase. The reaction mixture was preincubated at 37° Cent. for 15 minutes and the mitochondria added at zero time. The reaction was stopped after 15 minutes by icing and addition of 1.0 ml. of 0.5% HgCl<sub>2</sub>. After dilution with glass distilled water, assay for corticosterone was carried out by a modification of the sulfuric acid fluorescence method(58).

Desoxycorticosterone was obtained from the Sigma Chemical Co. The enzymes were purchased from either Calbiochem or C.F. Boehringer and Soehne, Mannheim, Germany. Nucleotides were purchased from the Sigma Chemical Co. and Calbiochem. C<sup>14</sup>-L-malic acid and H<sup>3</sup>-water were obtained from New England Nuclear. Bovine serum albumin was Pentex recrystallized. Para-iodobenzylmalonate was purchased from K & K Laboratories, Inc.



## ISOCITRIC DEHYDROGENASE

### 1. Distribution of Enzyme Activity

68% of the NADP-linked ICDH activity in the rat adrenal cell is localized in the 100,000g-60 min. soluble supernatant fraction. After sonication, the mitochondrial fraction shows 17% and the nuclei + waste fraction 7% of the total activity. The activities of each fraction are shown in Fig. 3 and Table I. The actual activity of the nuclei + waste fraction is probably less because this fraction included the mitochondrial washings. Latent enzyme activity was released by sonication. This process structurally disrupts whole cells, mitochondria, and other cellular constituents so that enzyme activity contained within equilibrates

TABLE I. SUBCELLULAR DISTRIBUTION OF NADP-LINKED ISOCITRIC DEHYDROGENASE

| FRACTION           | TOTAL ACTIVITY<br>μmoles NADPH/min. | % TOTAL ACTIVITY<br>RECOVERED | SPECIFIC ACTIVITY<br>μmoles <u>NADPH/min.</u><br>mgm. protein |
|--------------------|-------------------------------------|-------------------------------|---------------------------------------------------------------|
| Initial Homogenate | 5710                                | 100                           | 173                                                           |
| Nuclei + Waste     | 386                                 | 6.8                           | 49.5                                                          |
| Mitochondria       | 970                                 | 17.0                          | 104.                                                          |
| Microsomes         | 145                                 | 2.5                           | 34.5                                                          |
| Soluble            | 3860                                | 67.6                          | 391.                                                          |

The 5 subcellular fractions were isolated from the adrenal glands of 8 rats. Each fraction was diluted 6 fold and sonicated for 5 min. at 30 sec. intervals at 0-4° C. ICDH activity was measured spectrophotometrically at 340 mμ by the rate of reduction of 30 mM NADP in the presence of 8 mM isocitrate, 1 mM MnCl<sub>2</sub>, 0.1 M TRIS-HCl, and the sonicated enzyme.



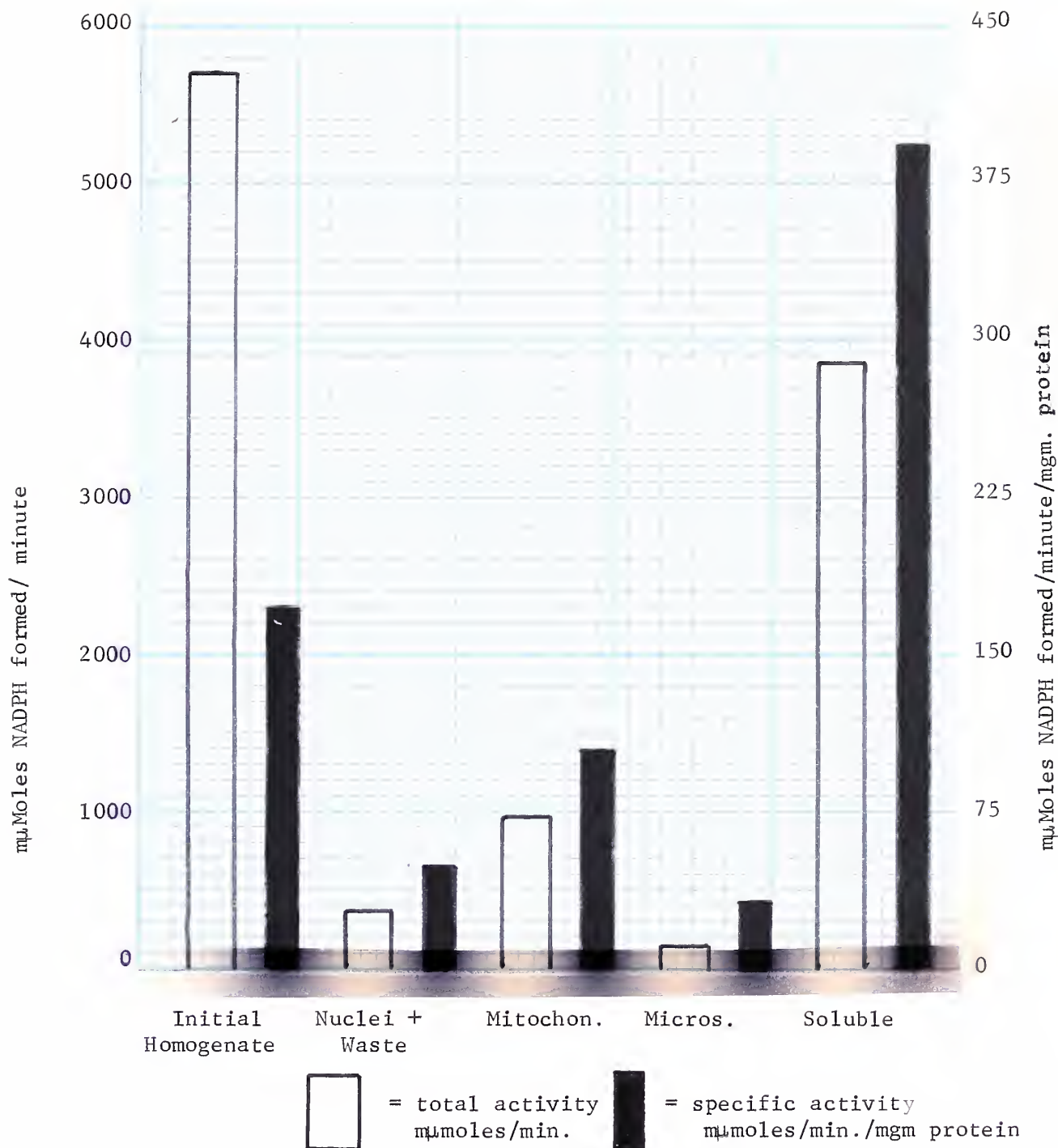


Figure 3. Subcellular Distribution of NADP-Linked Isocitric Dehydrogenase in the Rat Adrenal Cell.

The 5 subcellular fractions were isolated from the adrenal glands of 8 rats. Each fraction was diluted 6X and sonicated for 5 min. at 30 sec. intervals at 0-4° C. ICDH activity was measured spectrophotometrically at 340 mμ by the rate of reduction of 30 mM NADP in the presence of 8 mM isocitrate, 1 mM MnCl<sub>2</sub>, 0.1 M TRIS-HCl, and the sonicated enzyme fractions.



with the soluble phase. Note that ICDH is extremely abundant in the soluble cell fraction and seems to have about a 4 fold higher specific activity than the mitochondrial enzyme.

## 2. Kinetics of NADP-Linked Isocitric Dehydrogenase

The different locales of the soluble and mitochondrial ICDH suggests that these are two different enzymes. While this point was not extensively studied, further kinetic studies were done using the sonicated mitochondria and the soluble supernate. The soluble supernatant enzyme had a  $K_m$  for d-isocitrate of about 19  $\mu$ M whereas the  $K_m$  for the mitochondrial ICDH was 21-57  $\mu$ M. Unfortunately the enzymes were not purified sufficiently to do more accurate and detailed kinetics, but even with these data it can be seen that both enzymes have very small Michaelis constants for d-isocitrate. A Lineweaver-Burke plot of the soluble ICDH is shown in Fig. 4.

## 3. Isocitric Dehydrogenase Activity by $\alpha$ -Ketoglutarate Efflux

Another technique for assessing mitochondrial isocitrate oxidation is measurement of efflux of the product,  $\alpha$ -ketoglutarate. Most Krebs cycle intermediates are known to be permeable to the mitochondrial membrane to varying degrees. It was found that  $\alpha$ -ketoglutarate will diffuse out of the mitochondria into the incubation medium at a constant rate during isocitrate oxidation. Fig. 5 shows the rate of  $\alpha$ -ketoglutarate efflux from mitochondria utilizing exogenous isocitrate in the absence of inhibitors of oxidative phosphorylation. The presence of 1 mM arsenite inhibits the oxidation of  $\alpha$ -ketoglutarate to succinate so that the resulting accumulation



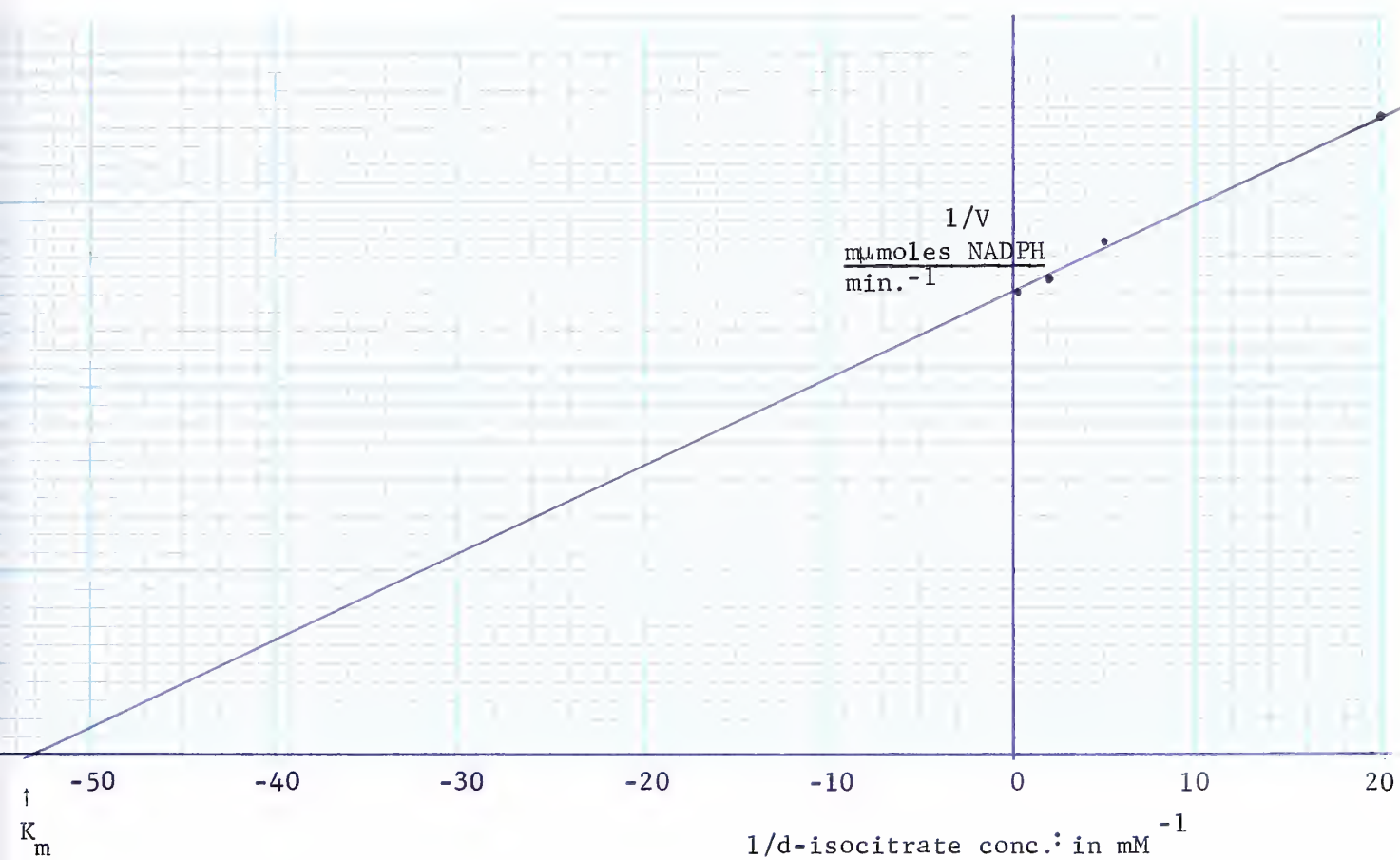


Figure 4. Lineweaver-Burke plot of Soluble Fraction ICDH

Soluble fraction ICDH activity was measured spectrophotometrically at 340  $\text{m}\mu$  by rate of NADPH formation in the presence of 1 mM  $\text{MnCl}_2$ , 0.1 M TRIS-HCl, 30 mM NADP, variable isocitrate concentration, and the sonicated enzyme.

Approximate  $K_m$  for d-isocitrate = 0.019 mM



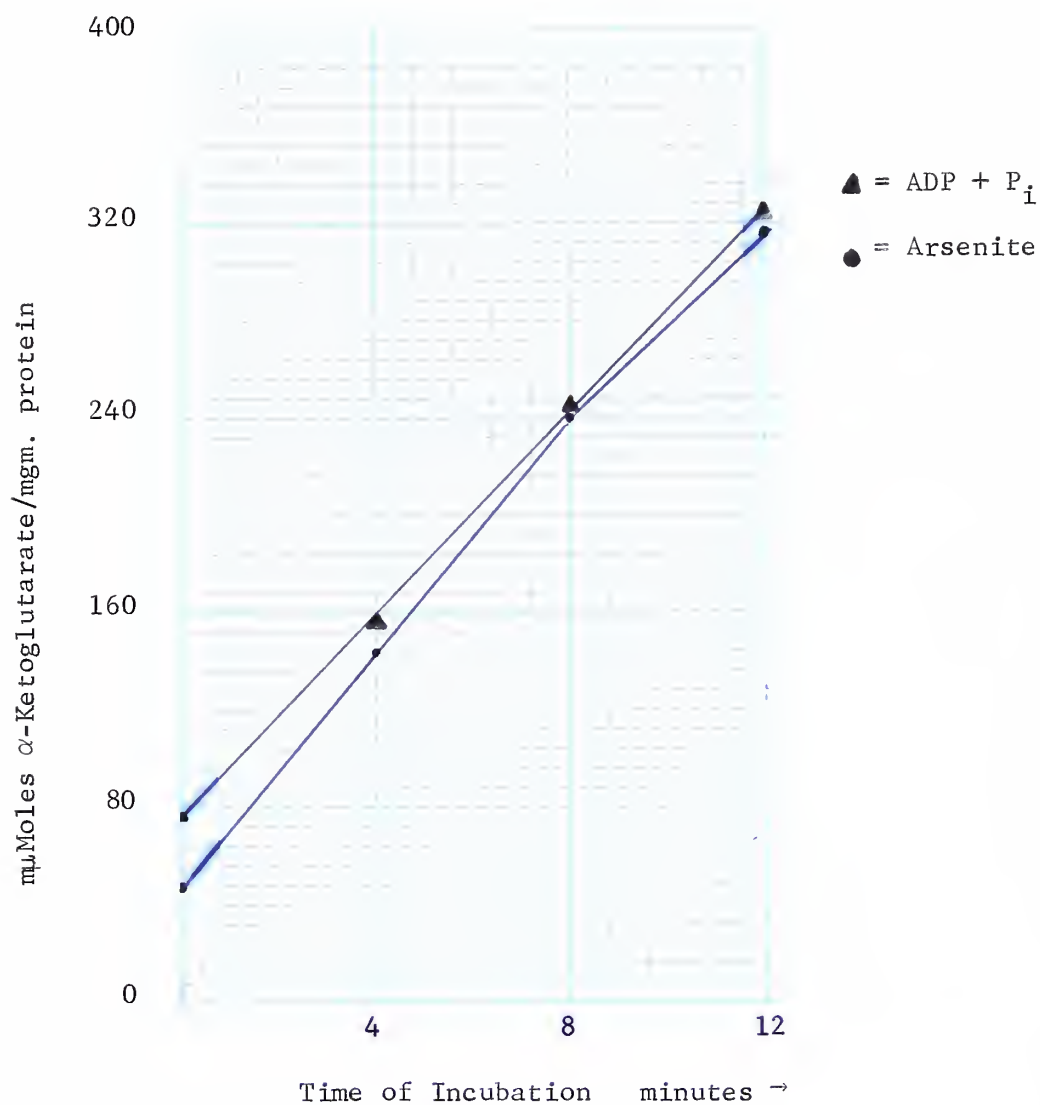


Figure 5. Effects of ADP + P<sub>i</sub> and Arsenite upon α-Ketoglutarate Efflux

Flasks were incubated at 37° C. for the times shown. Flask contents included: 10 mM isocitrate, 2 mM P<sub>i</sub>, 0.4 mM ADP, 1 mM arsenite, and 0.5 mgm. mitochondrial protein/ml. α-Ketoglutarate was measured by enzymatic methods.



of  $\alpha$ -ketoglutarate should enhance diffusion out of the mitochondria. In the other flask the presence of  $\text{ADP} + \text{P}_i$  increased the rate of isocitrate oxidation by providing the necessary substrates for oxidative phosphorylation. Note that the rate of  $\alpha$ -ketoglutarate efflux is not only reasonably constant but is not augmented by arsenite. This may mean that during isocitrate oxidation  $\alpha$ -ketoglutarate is produced in sufficient quantity to completely saturate the mechanism which mediates  $\alpha$ -ketoglutarate efflux. An enhancement of efflux would therefore not be expected with a greater quantity of intramitochondrial  $\alpha$ -ketoglutarate formed by the effect of arsenite.

Fig. 6 demonstrates the effect of steroid hydroxylation on the rate of  $\alpha$ -ketoglutarate efflux from KCN-inhibited mitochondria. NADPH produced by NADP-linked ICDH is available to the P450 pathway. In the presence of adequate steroid substrate this pathway therefore acts as an electron sink by reoxidizing the NADPH made available by isocitrate oxidation. 2 mM KCN was added to inhibit cytochrome oxidase and thus diminish the contribution of the NAD-linked malic dehydrogenase. In addition, 1 mM arsenite is present to maximize  $\alpha$ -ketoglutarate efflux. Fig. 6 shows that the presence of DOC stimulated  $\alpha$ -ketoglutarate efflux originating from the NADP-linked pathway.

According to Chappell, isocitrate entry into the mitochondria requires the additional presence of malate and inorganic phosphate(59). Fig. 7 shows  $\alpha$ -ketoglutarate efflux  $\pm$  DOC and the effect of malate and  $\text{P}_i$ . There does not seem to be any appreciable difference in the rate of  $\alpha$ -ketoglutarate efflux before or after the addition of these 2 factors. Under these



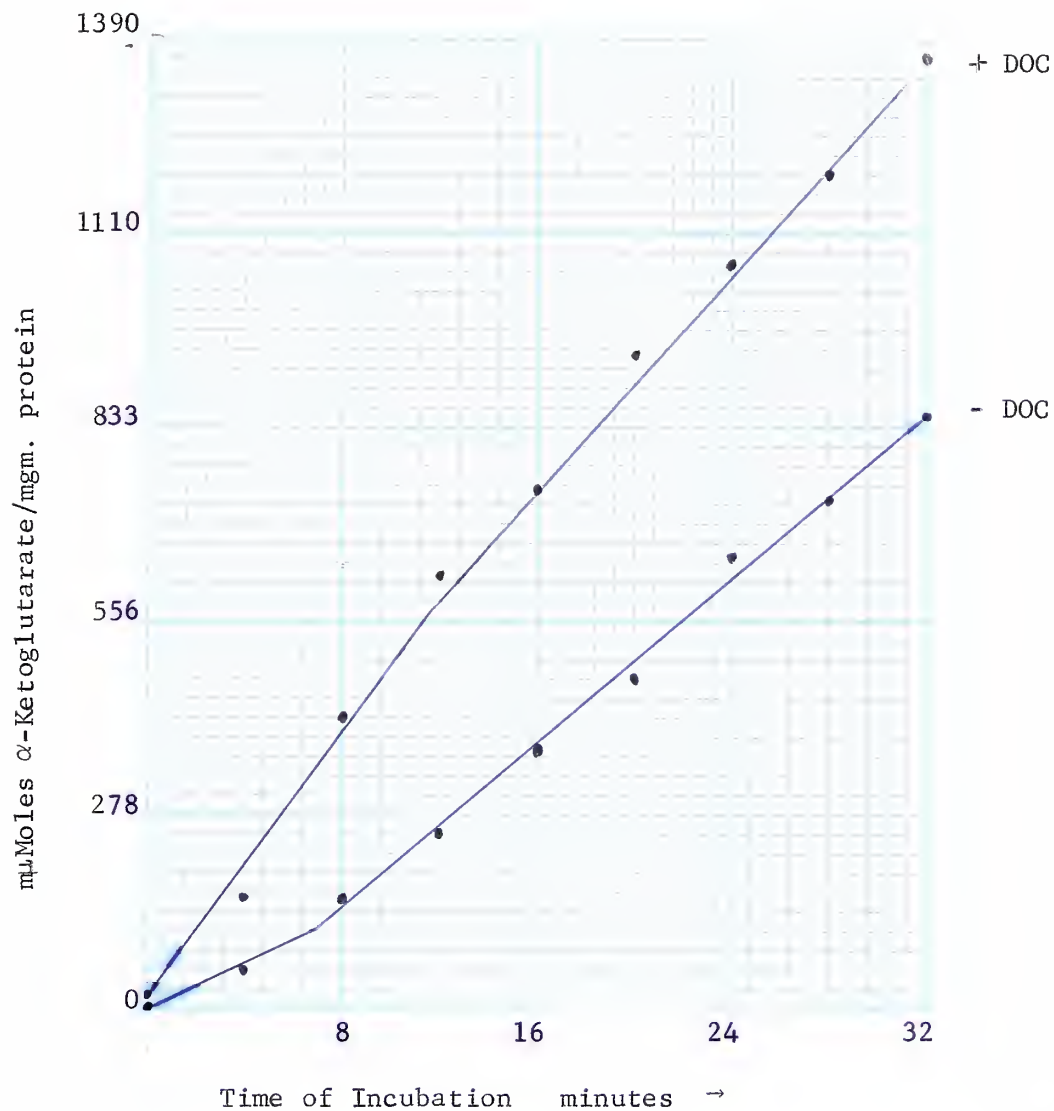


Figure 6. Effect of DOC upon  $\alpha$ -Ketoglutarate Efflux from KCN Inhibited Mitochondria During Isocitrate Support.

Two flasks were incubated at  $37^{\circ}$  C. with  $\frac{1}{2}$  ml. aliquots removed at times shown. Incubation mixture contained 10 mM isocitrate, 2 mM KCN, 1 mM arsenite,  $\pm 100 \mu\text{M}$  DOC, and 0.36 mgm. mitochondrial protein/ml. Reaction was stopped by centrifuging down the mitochondria. Assay of  $\alpha$ -ketoglutarate in the supernatant fluid was carried out by enzymatic methods.



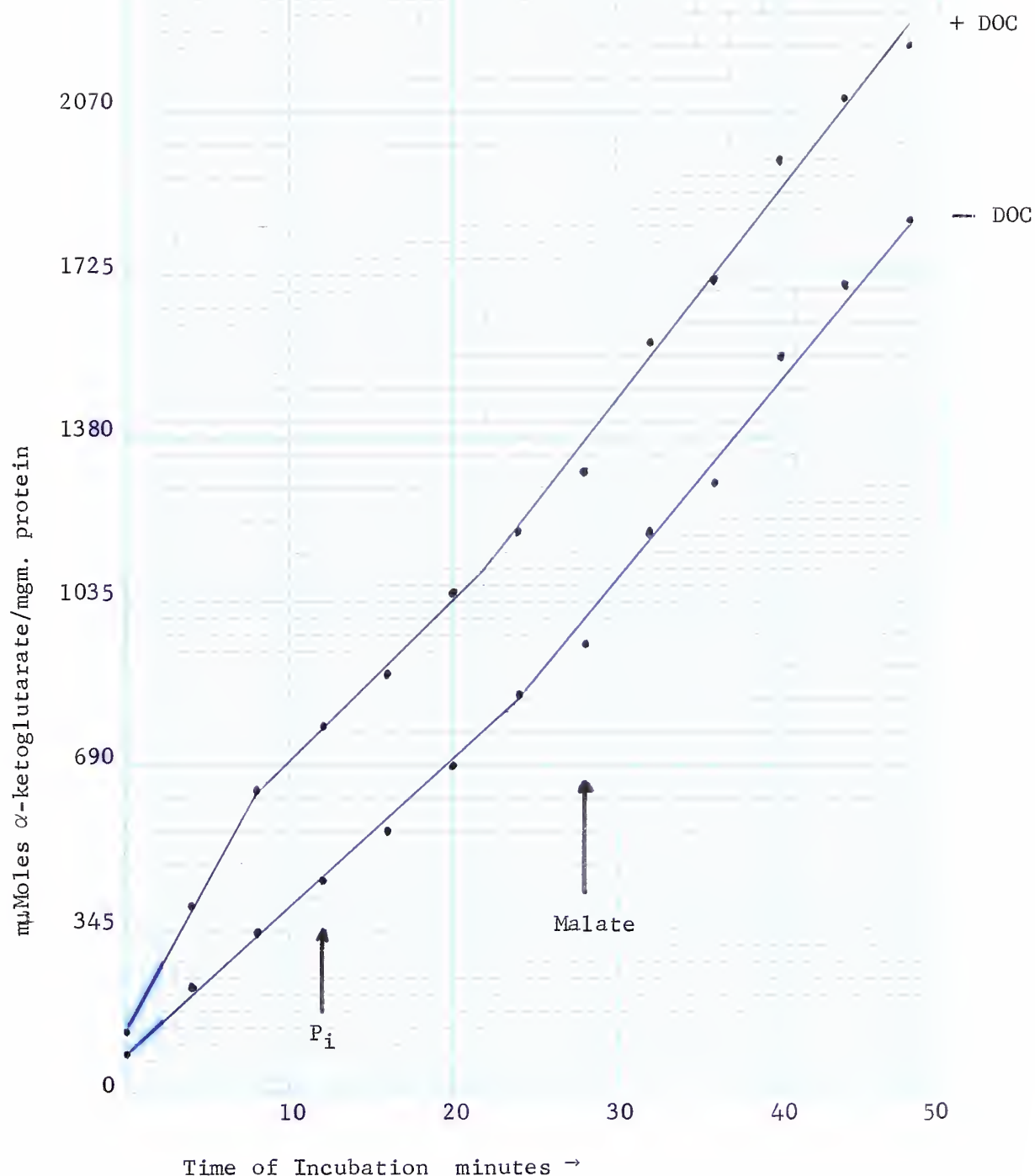


Figure 7. Effect of  $P_i$  and Malate on  $\pm$  DOC Stimulated  $\alpha$ -Ketoglutarate Efflux from Isocitrate Supported Mitochondria.

Two flasks were incubated at  $37^\circ$  C. with  $\frac{1}{2}$  ml. aliquots removed at 4 min. intervals. Final incubation mixture contained 10 mM isocitrate, 1 mM arsenite,  $\pm$  100  $\mu$ M DOC, 2 mM  $P_i$ , 0.2 mM malate, and 0.435 mgm. mitochondrial protein/ml. The reaction was stopped by centrifuging down the mitochondria and assaying the supernate.  $\alpha$ -ketoglutarate was measured by enzymatic methods.



conditions, no evidence was found for enhanced isocitrate entry based on  $\alpha$ -ketoglutarate efflux.

#### 4. Partial Purification of ICDH and Shuttle Experiment

The presence of a highly active NADP-linked ICDH in the soluble cell fraction and evidence that  $\alpha$ -ketoglutarate produced during isocitrate oxidation diffused out of the mitochondria led to speculation regarding a possible isocitrate shuttle system. If such a system were operative, intramitochondrial NADP-linked isocitrate oxidation would produce intramitochondrial NADPH for steroid hydroxylation. The  $\alpha$ -ketoglutarate also formed could then diffuse into the soluble cell fraction. The highly active extramitochondrial NADP-linked ICDH could utilize NADPH and  $\text{HCO}_3^-$  to reform isocitrate. In this manner, isocitrate could re-enter the mitochondria and effect a net transfer of reducing equivalents across the mitochondrial membrane. Experiments designed to demonstrate this possibility were attempted. A crude purification of soluble NADP-linked ICDH activity was undertaken as follows: The fractions from the 100,000g-60 min. supernatant fluid precipitating between 58 and 75% saturation with ammonium sulfate were dialyzed against 0.1 M TRIS-HCl buffer to remove  $\text{NH}_4^+$ . These fractions, which possessed the highest NADP-linked ICDH activity, were incubated with mitochondria, DOC, and the reactants necessary for the conversion of  $\alpha$ -ketoglutarate to isocitrate. However, no steroid hydroxylation above control rates could be demonstrated. This experiment was repeated on several occasions, but each time negative results were obtained.

The failure of this shuttle to support steroid hydroxylation in vitro does not speak against its existence in vivo. Despite the fact that the

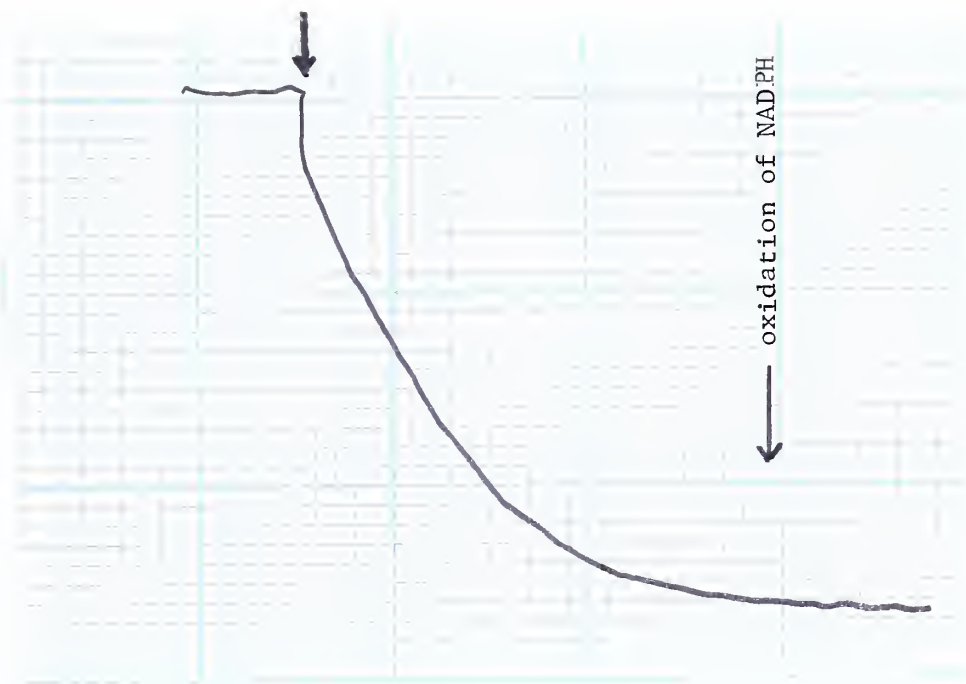


reverse reaction is kinetically unfavorable, we were able to demonstrate spectrophotometrically that commercial NADP-linked ICDH catalyzed the oxidation of NADPH in the presence of  $\alpha$ -ketoglutarate (Fig. 8). Note that the reverse reaction is completely dependent on  $\text{HCO}_3^-$  and NADPH. It seems therefore that the reverse reaction is biochemically possible, but that our in vitro incubation was not sufficiently sensitive to detect the small amount of isocitrate formed. At the present time, we cannot assess the degree to which this isocitrate shuttle is operative in vivo.

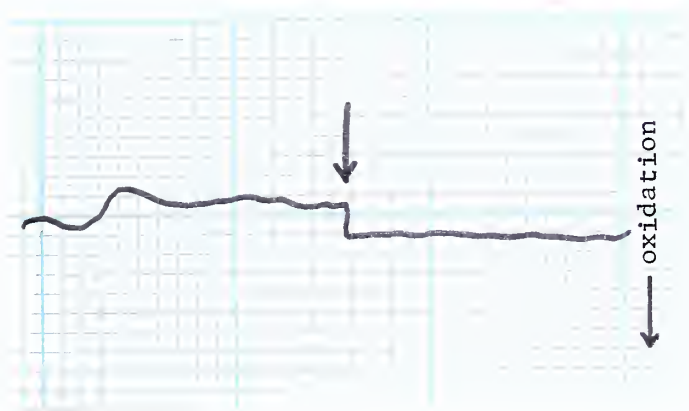
In summary, the following facts may be derived from the results of the preceding experiments:

- 1) NADP-linked ICDH is found both in the mitochondria and in the soluble fraction of the rat adrenal cell.
- 2) ICDH is a highly active mitochondrial enzyme capable of supporting high rates of steroid hydroxylation.
- 3)  $\alpha$ -ketoglutarate formed by NADP-linked ICDH can diffuse out of the mitochondria even in the absence of arsenite. This efflux is augmented by steroid hydroxylation.
- 4) ICDH can catalyze the reverse reaction (oxidize NADPH and form isocitrate), but under our incubation conditions this does not occur at a rate sufficient to support steroid hydroxylation.

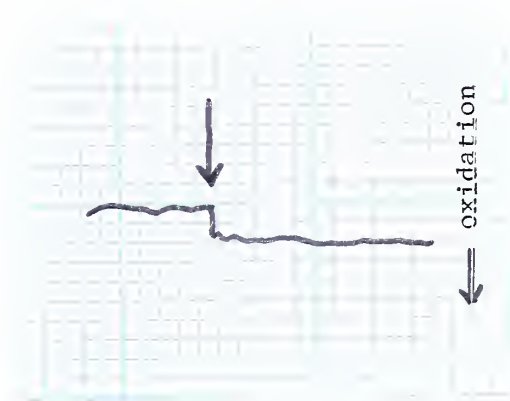




1. Complete system



2. w/out  $\text{HCO}_3^-$



3. NADH in place of NADPH

Figure 8. Spectrophotometric oxidation of NADPH at 340  $\mu$  by commercial NADP-linked ICDH.

Cuvette contained 10 mM  $\alpha$ -ketoglutarate, 10 mM  $\text{Mn}^{++}$ , 1 mM arsenite,  $\pm$  NADPH (NADH added in place of NADPH),  $\pm$  50 mM  $\text{HCO}_3^-$ , and the commercial enzyme. The smallest squares on the paper represent 31.1  $\mu$ moles of NADPH. The reaction was started by the addition of ICDH (arrow), and oxidation occurs in the direction shown.



## MALIC ENZYME

### 1. Distribution of Enzyme Activity

Based on the spectrophotometric reduction of NADP in the presence of malate, 94% of the malic enzyme activity in the rat adrenal cell is localized in the 100,000g-60 min. soluble supernatant fraction. Following sonication the microsomes demonstrate about 1%, the nuclei 0.6%, and the mitochondria 1.3% of the enzyme activity (Table II and Fig. 9). Unlike ICDH, mitochondrial ME activity was not spectrophotometrically detectable after sonication. The specific activity of the enzyme was highest in the soluble fraction. Negligible amounts were found in the nuclei, microsomes, and mitochondria. Of significance is the 17 fold higher specific

TABLE II. SUBCELLULAR DISTRIBUTION OF MALIC ENZYME ACTIVITY

| FRACTION           | TOTAL ACTIVITY<br>$\mu$ moles NADPH/min. | % TOTAL ACTIVITY<br>RECOVERED | SPECIFIC ACTIVITY<br>$\frac{\mu\text{moles NADPH/min}}{\text{mgm. protein}}$ |
|--------------------|------------------------------------------|-------------------------------|------------------------------------------------------------------------------|
| Initial homogenage | 815.                                     | 100                           | 10.2                                                                         |
| Nuclei             | 5.25                                     | 0.64                          | 0.757                                                                        |
| Mitochondria       | 10.5                                     | 1.29                          | 0.734                                                                        |
| Microsomes         | 8.67                                     | 1.06                          | 1.59                                                                         |
| Soluble            | 763.                                     | 93.7                          | 23.2                                                                         |

The 5 subcellular fractions were isolated from the adrenals of 10 rats. Each fraction was diluted 4 fold with 0.25 M sucrose and sonicated for 5 min. at 30 sec. intervals at 0-4° C. NADP-linked ME activity was measured spectrophotometrically at 340 m $\mu$  by the rate of reduction of NADP in the presence of 10 mM malate, 10 mM MgCl<sub>2</sub>, 0.1 M TRIS-HCl, and NADP.



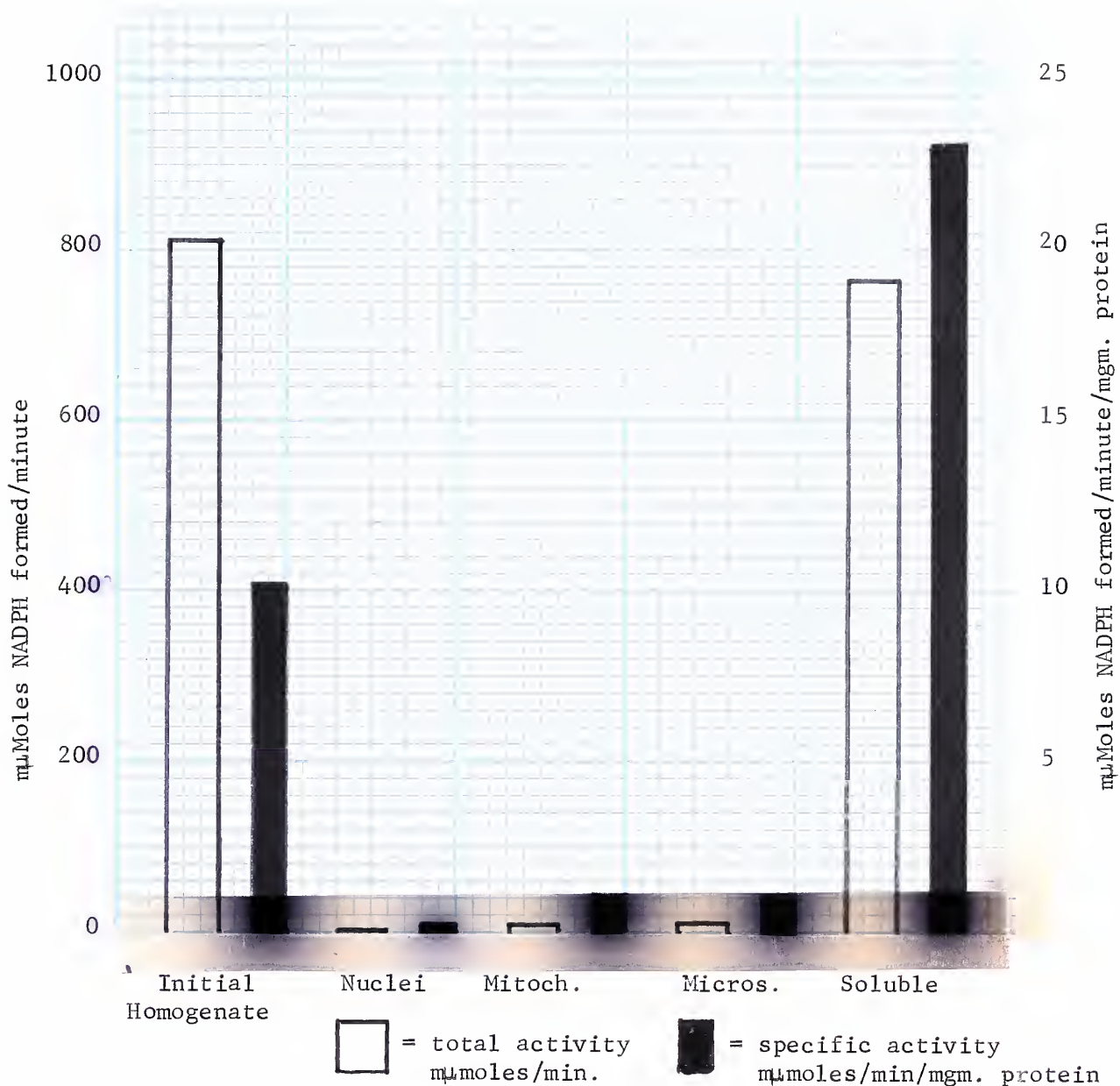


Figure 9. Subcellular Distribution of Malic Enzyme Activity in the Rat Adrenal Cell

The 5 subcellular fractions were isolated from the adrenals of 10 rats. Each fraction was diluted 4X with 0.25 M Sucrose and sonicated for 5 min. at 30 sec. intervals at 0-4° C. NADP-linked ME activity was spectrophotometrically measured at 340 mμ by rate of NADP reduction in the presence of 10 mM malate, 10 mM MgCl<sub>2</sub>, 0.1 M TRIS-HCl, and NADP.



activity of NADP-linked ICDH over ME in the soluble fraction (Tables 1 and 2). The rat adrenal cell thus seems much more capable of utilizing isocitrate for extramitochondrial NADPH formation than malate.

## 2. Malic Enzyme Activity by Pyruvate Efflux

Despite the fact that mitochondrial ME activity was not apparent spectrophotometrically, we observed the accumulation of pyruvate during in vitro incubations of the 100,000g-60 min. supernatant fluid from sonicated mitochondria. Pyruvate production versus the length of incubation is shown in Fig. 10.

Using intact rat adrenal mitochondria, Sauer has shown that malate supported steroid 11 $\beta$  hydroxylation and pyruvate efflux are augmented by the addition of arsenate or  $P_i$  (34). Thus it seemed that the  $P_i$  stimulation of pyruvate efflux would result in a situation analagous to  $\alpha$ -ketoglutarate efflux during isocitrate oxidation. Fig. 11 shows an experiment in which the effect of arsenite upon  $P_i$  stimulated pyruvate efflux was assessed. Conditions were adjusted for maximal pyruvate efflux: 1) DOC was present to act as substrate for steroid hydroxylation. 2) KCN was added to inhibit the respiratory chain and to decrease the contribution by the NAD-linked malate dehydrogenase (pyruvate might also arise from oxaloacetate, one product of MDH), and 3) Arsenite was added to inhibit further oxidation of pyruvate. Note that the addition of  $P_i$  (arrow) caused a marked stimulation of pyruvate efflux from the mitochondria. Also, pyruvate accumulated even in the absence of arsenite but 1 mM arsenite significantly augmented the rate of efflux. Thus we have demonstrated a ME activity based on the efflux of pyruvate from intact mitochondria but were unable to show NADP reduction



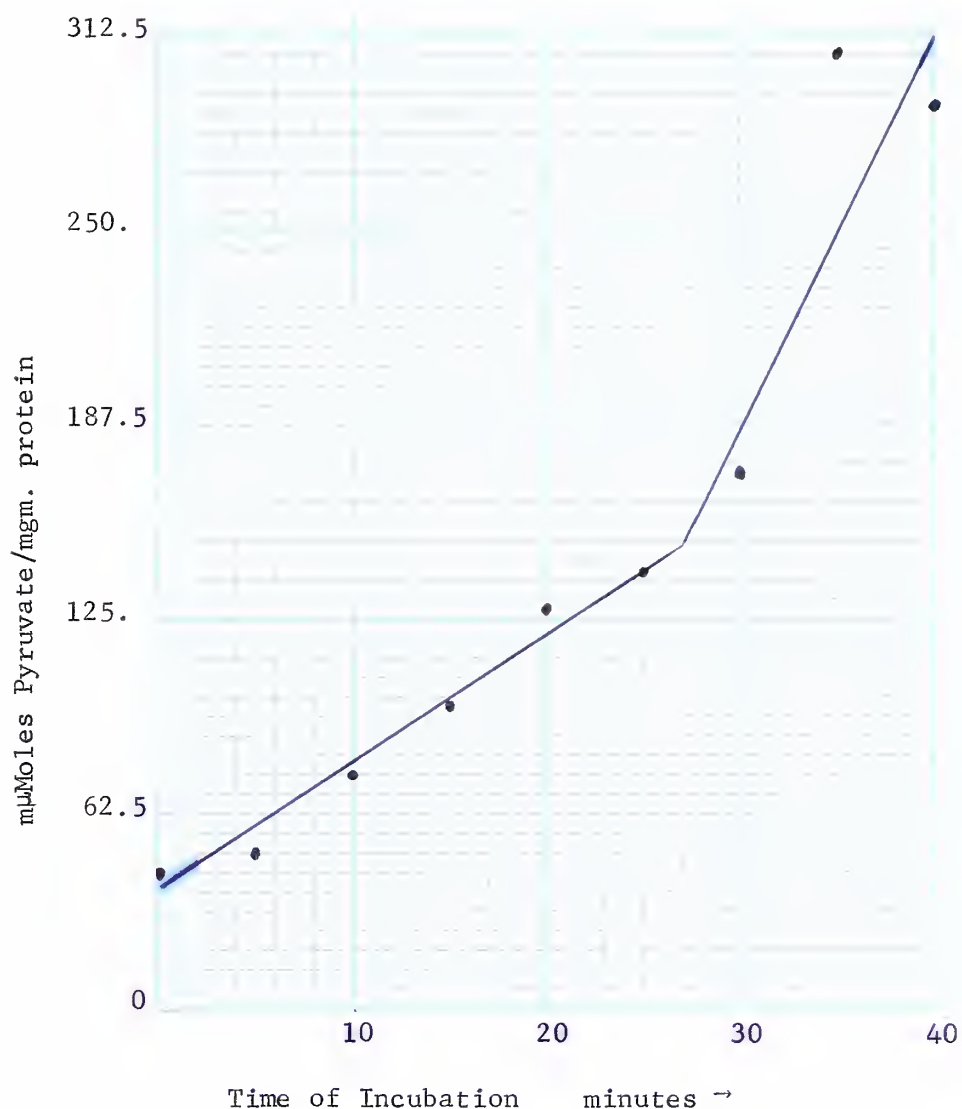


Figure 10. Malic Enzyme Activity in Supernatant Fluid of Sonicated Mitochondria

One flask was incubated at 37° C. with 1.0 ml. aliquots removed at 5 min. intervals. Flask contents included: incubation medium, 10 mM malate, 10 mM MgCl<sub>2</sub>, 2 mM KCN, 0.05 mM NADP, and 0.16 mgm. mitochondrial protein/ml. Incubation was begun with the addition of malate. The reaction was stopped by adding 0.1 ml of 70% Perchloric acid to the 1.0 ml aliquots. After neutralizing with KOH, pyruvate was measured enzymatically.



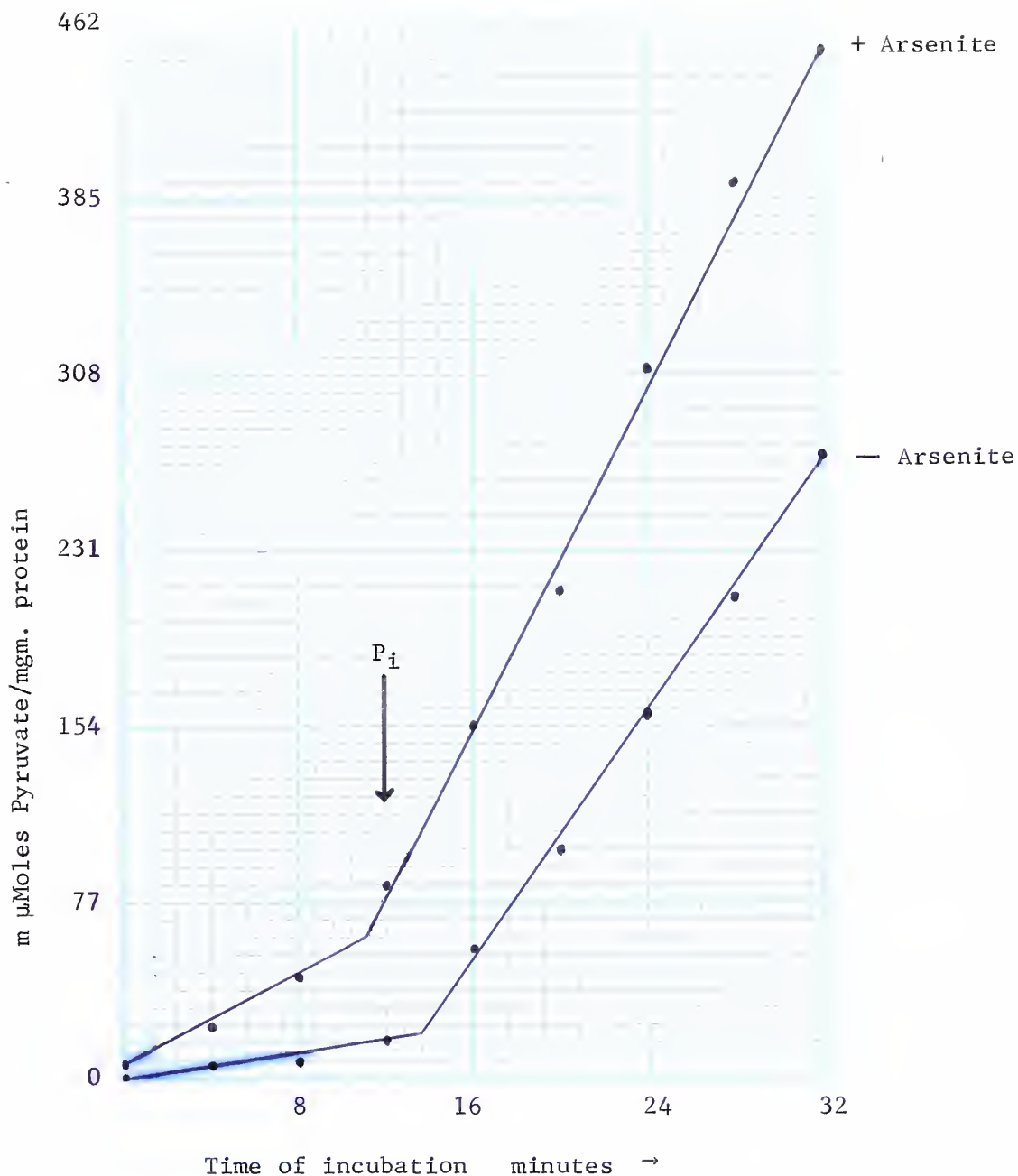


Figure 11. Effect of Arsenite on DOC and  $P_i$  Stimulated Pyruvate Efflux from Malate Supported Mitochondria

Two flasks were incubated at  $37^\circ \text{C}$ . with aliquots removed at 4 min. intervals. Reagents included incubation medium, 10 mM malate, 100  $\mu\text{M}$  DOC, 2 mM  $P_i$ , 2 mM KCN,  $\pm 1 \text{ mM}$  arsenite, and 0.65 mgm. mitochondrial protein/ml. Malate was added at the start of the reaction, and  $P_i$  was added as shown. The reaction was stopped by centrifuging down the mitochondria. Pyruvate was measured in the supernatant fluid by enzymatic methods.



spectrophotometrically in sonicated mitochondria.

The property of 2 mM  $P_i$  to stimulate pyruvate efflux was a reproducible phenomenon which occurred in 9 different experiments. In Fig. 12 the effect of variable  $P_i$  concentration upon pyruvate efflux is shown. Note the very small  $K_m$  for this stimulation of efflux. Also note that about 80% of maximal velocity is attained using a  $P_i$  concentration of 2 mM.

Presumably,  $P_i$  activates a membrane transport system for malate so that the augmented efflux results from a greater amount of substrate being delivered to the mitochondrial matrix space. That pyruvate efflux is directly dependent upon the presence of malate is indicated by an experiment in which malate was added to 1 of 2 flasks (Fig. 13). Here, 2 mM  $P_i$  was present before the malate addition (arrow). Note that there was no accumulation of pyruvate until malate was added, and the flask without malate showed no pyruvate efflux at any time. This experiment proves that pyruvate formation is completely dependent upon malate.

Recently, Robinson and Williams (60) reported that para-iodobenzyl-malonate (p-IBM), a structural analogue of malic acid, was effective in the inhibition of dicarboxylic acid membrane transport systems. Experiments designed to test the efficacy of p-IBM in the inhibition of malate entry are shown in Fig. 14 and 15. In Fig. 14, mitochondrial pyruvate efflux is shown using 2 flasks, one of which contains 20 mM p-IBM. Note that upon addition of  $P_i$  pyruvate efflux from the flask containing p-IBM was inhibited relative to the pyruvate efflux seen in the control flask. This suggests that p-IBM may inhibit the entry of malate into the mitochondria.



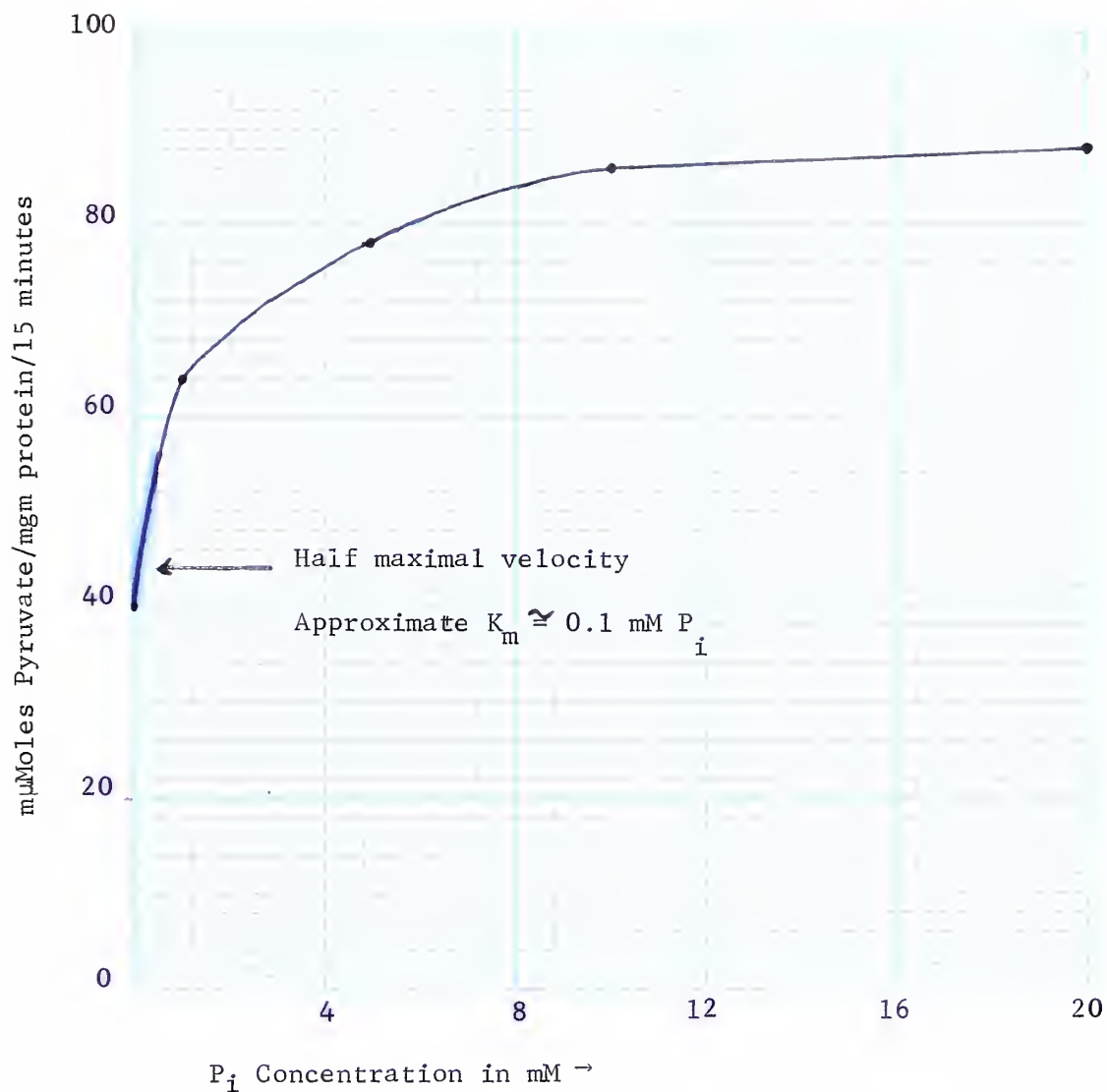


Figure 12. The Effect of  $P_i$  Concentration upon Pyruvate Efflux from Intact Mitochondria.

Flasks were incubated for 15 min. at  $37^\circ \text{C}$ . Contents included incubation medium, 10 mM malate, 2 mM KCN, 0.425 mgm. mitochondrial protein/ml., and variable  $P_i$  concentration.  $P_i$  was added at the beginning of the incubation. Pyruvate was measured by enzymatic methods after stopping the reaction by centrifuging down the mitochondria.



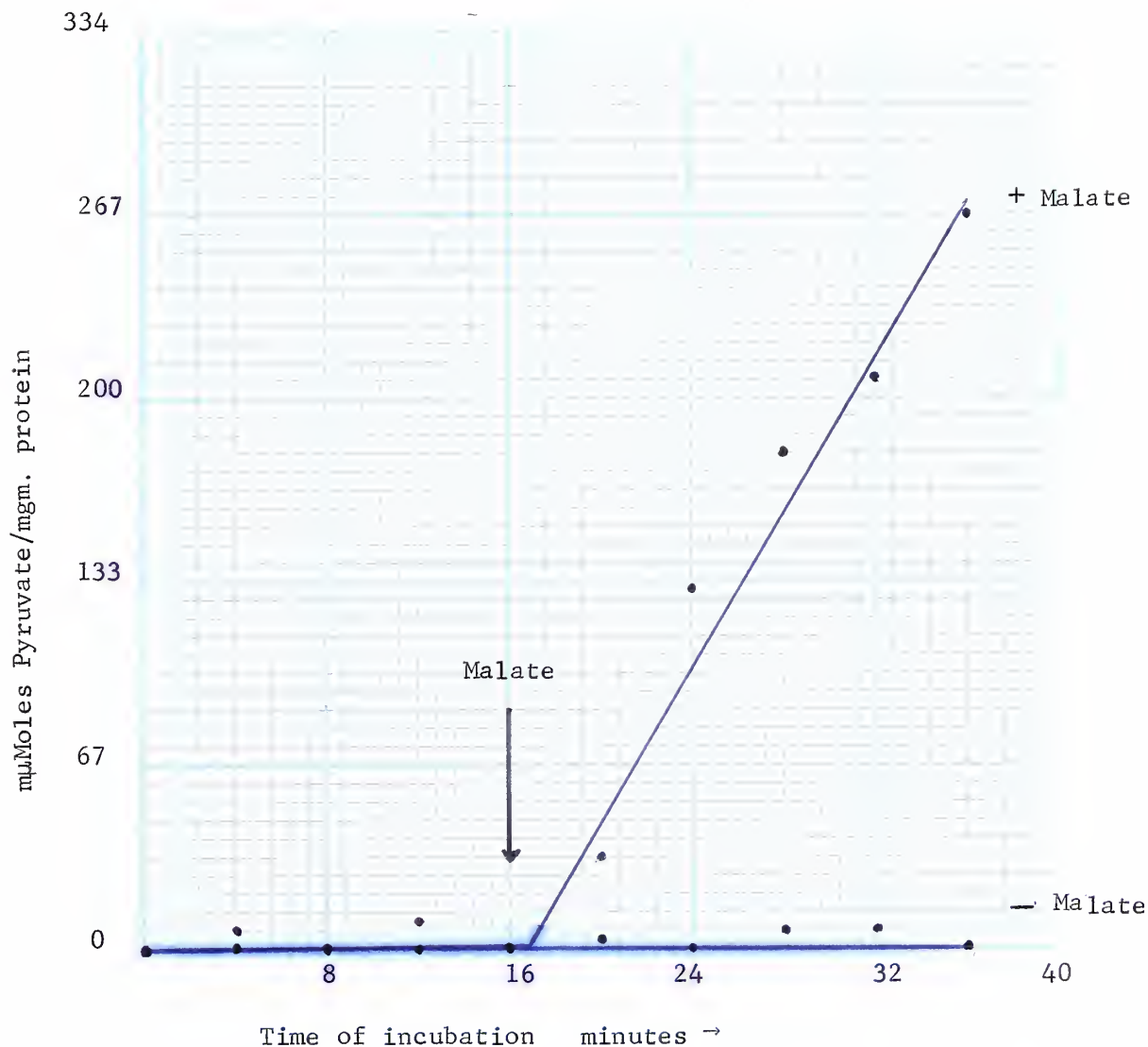


Figure 13. Dependence of  $P_i$  Stimulated Pyruvate Efflux upon Malate

Two flasks were incubated at  $37^\circ$  C. with the following reagents: incubation medium, 2 mM KCN, 2 mM  $P_i$ , 0.3 mgm. mitochondrial protein/ml, and  $\pm$  10 mM malate (added at arrow). Aliquots were removed at 4 min. intervals and the reaction stopped by centrifuging down the mitochondria. The pyruvate in the supernate was measured enzymatically.



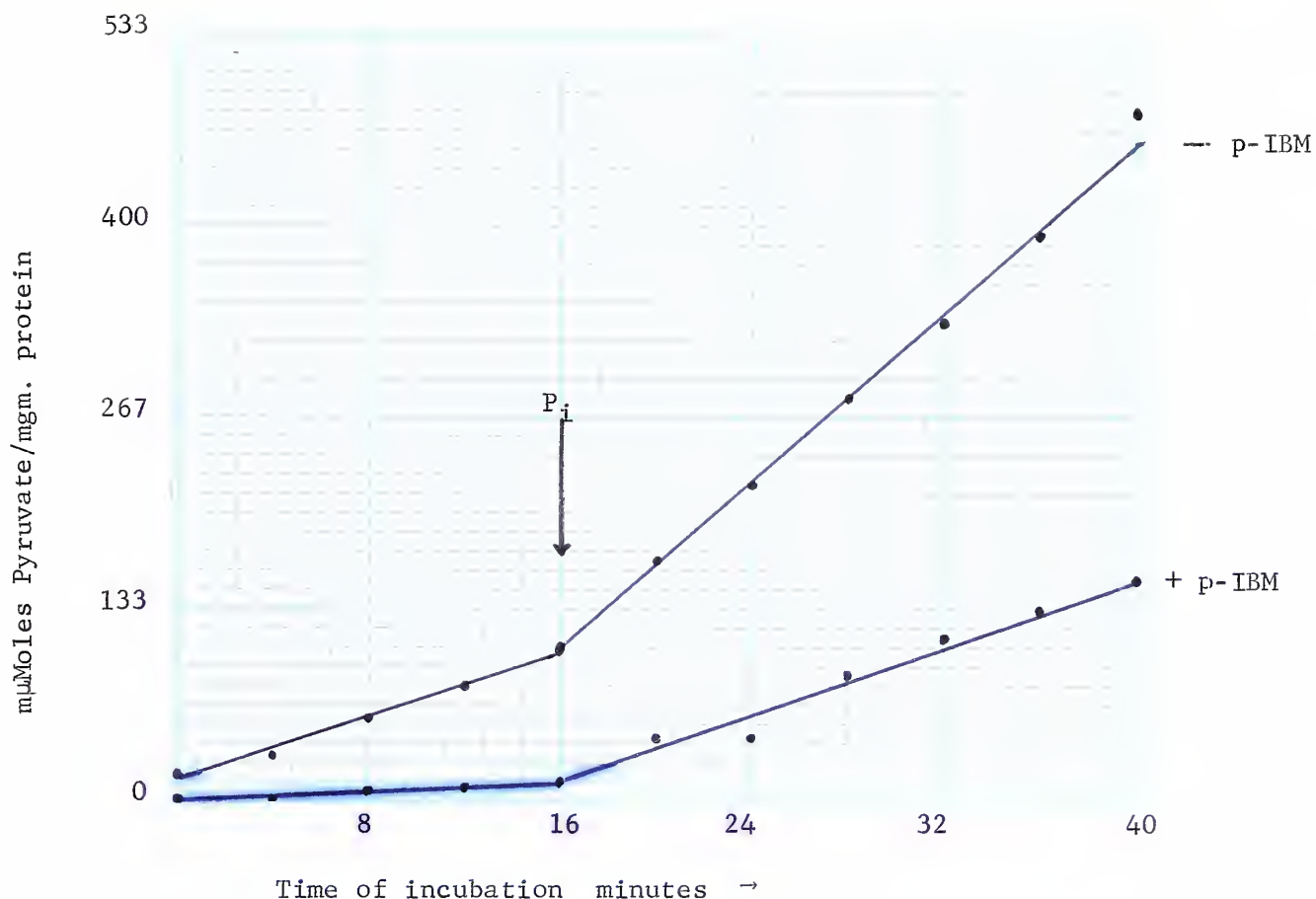


Figure 14. Inhibitory Effect of p-IBM upon  $P_i$  Stimulated Pyruvate Efflux

2 flasks were incubated at  $37^{\circ}\text{C}$ . with the following contents: incubation medium, 10 mM malate, 2 mM KCN,  $\pm$  20 mM p-IBM, 2 mM  $P_i$ , and 0.60 mgm. mitochondrial protein/ml. The reaction was started by addition of malate, and  $P_i$  was added as shown. Pyruvate was measured by enzymatic methods.



Fig. 15 demonstrates the effect of p-IBM added at a time when entry and oxidation of malate and pyruvate efflux are occurring. 20 mM p-IBM caused almost complete inhibition of further pyruvate efflux within 3 minutes after addition. If p-IBM indeed acts on the membrane transport system for dicarboxylic acids, then it seems logical to deduce that pyruvate efflux ceases when the intramitochondrial pool of malate becomes depleted following the inhibition of malate transport and entry.

The previous experiments have provided evidence for the presence of mitochondrial malic enzyme activity.  $P_i$  has been shown to enhance pyruvate efflux, and this in turn is augmented by 1 mM arsenite and DOC and is completely dependent upon malate. The results with p-IBM suggest that malate enters the mitochondria via a transport system which can be inhibited by structural analogues. These experiments, however, demonstrate only indirectly the entry of malate into the mitochondria by showing changes in efflux of the ME reaction product.

In an effort to demonstrate the direct entry of malate, mitochondrial swelling experiments were carried out using  $\text{NH}_4$ -malate. Chappell(59) has shown that swelling occurs in the following manner:  $\text{NH}_4^+$  dissociates into  $\text{H}^+$  and  $\text{NH}_3$ . The latter readily diffuses into the mitochondria where it is protonated to reform the relatively nondiffusible  $\text{NH}_4^+$ . Malate then enters the mitochondria in exchange for the  $\text{OH}^-$  which, together with the  $\text{H}^+$  used to protonate  $\text{NH}_3$ , arose from hydrolysis. This influx of  $\text{NH}_4$ -malate exerts a solute drag effect and causes influx of water and consequent mitochondrial swelling. This is detectable spectrophotometrically at a light wavelength of 600 m $\mu$ . Fig. 16 shows a typical example where



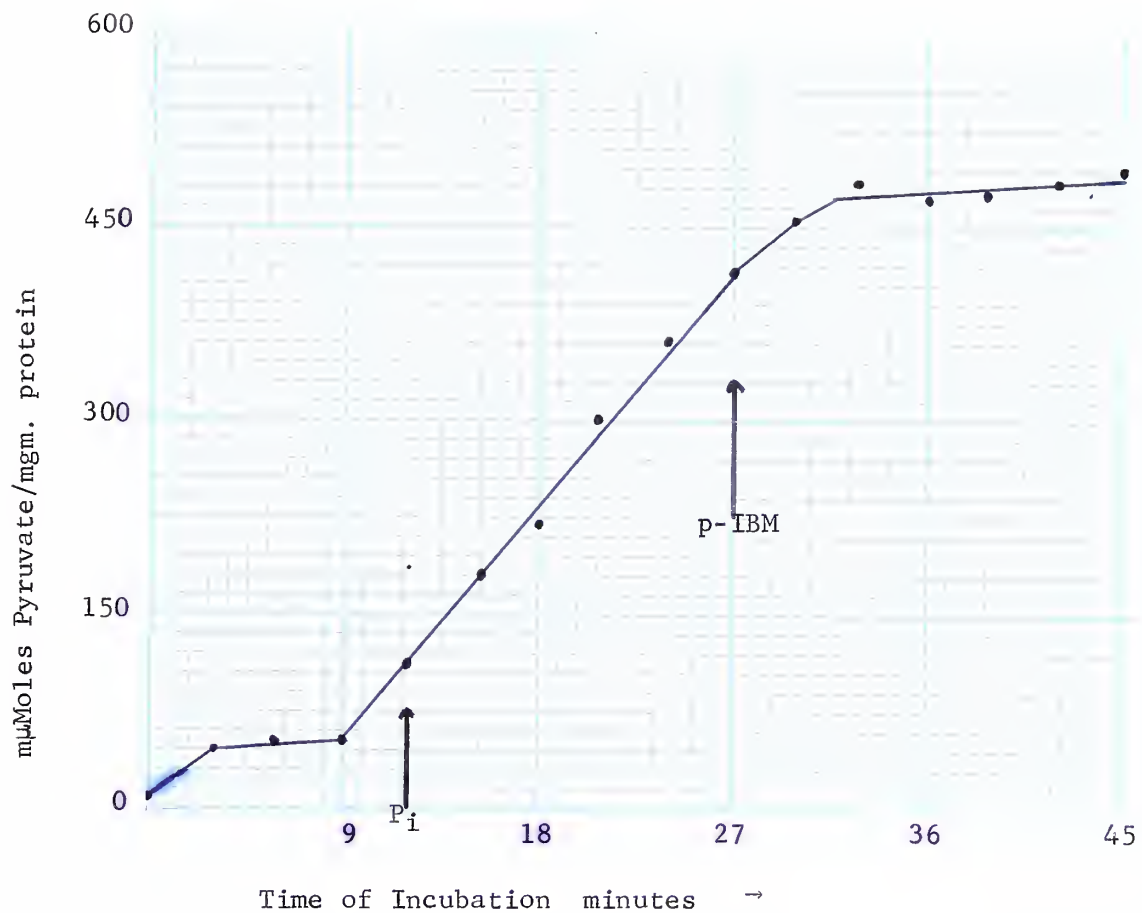


Fig. 15. Effect of p-IBM on  $P_i$  Stimulated Pyruvate Efflux from Malate Supported Mitochondria

One flask was incubated at  $37^\circ$  C. with the following contents: incubation medium, 10 mM malate, 2 mM KCN, 2 mM  $P_i$ , 20 mM p-IBM, and 0.4 mgm. mitochondrial protein/ml. The reaction was started by the addition of malate;  $P_i$  and p-IBM were added as shown. The reaction was stopped by centrifuging down the mitochondria. Pyruvate in the supernate was measured by enzymatic methods.



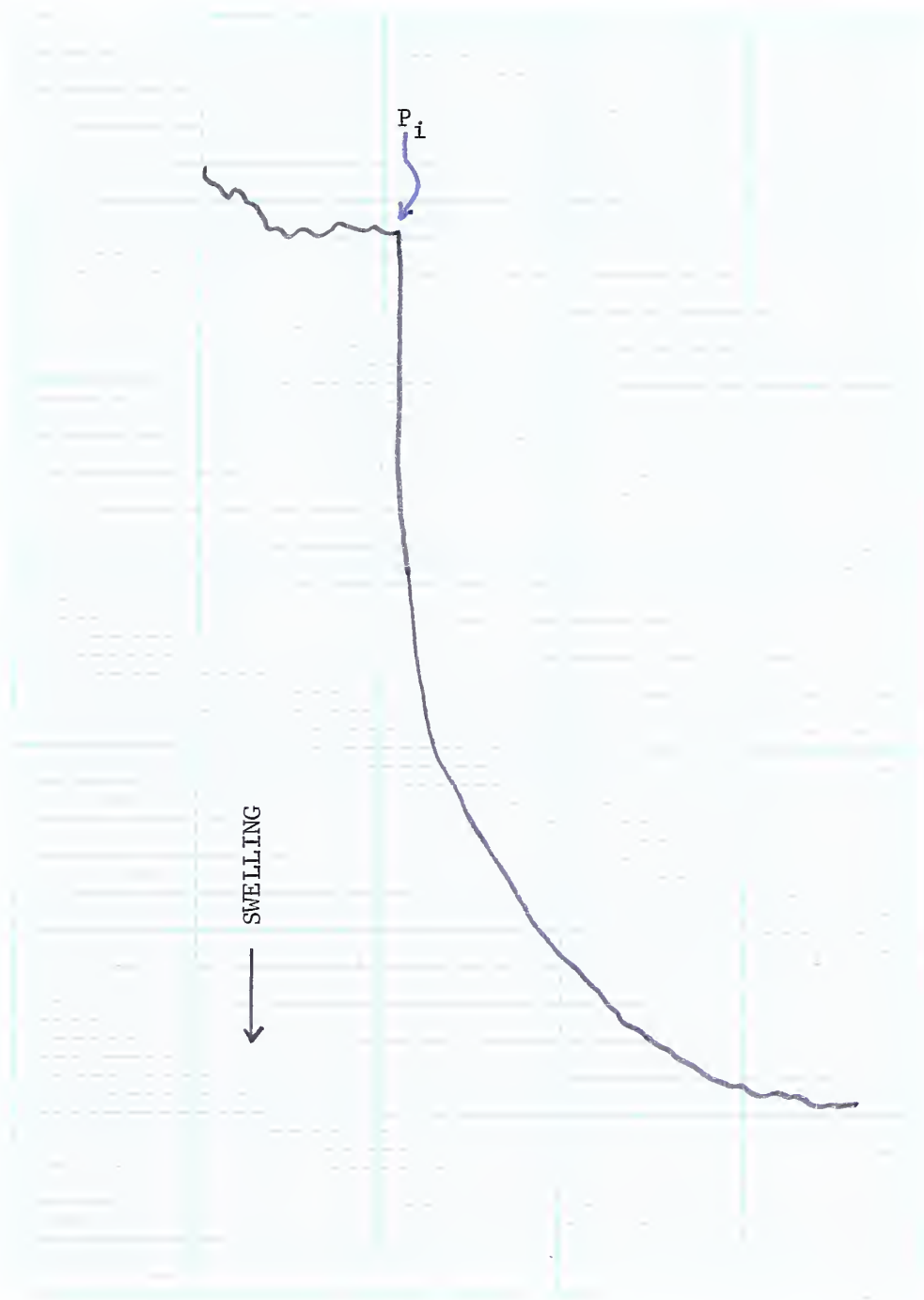


Figure 16. Spectrophotometric detection of mitochondrial swelling during  $\text{NH}_4$ -malate incubation.

Adrenal mitochondria were suspended in a medium containing 90 mM  $\text{NH}_4$ -malate and  $10^{-5}$  M rotenone. 6 mM  $\text{P}_i$  was added (arrow) to initiate the swelling detected at a wavelength of 600 mμ. The cuvette contained about 0.12 mgm. of mitochondrial protein.



$P_i$  stimulated influx of  $NH_4$ -malate resulted in mitochondrial swelling evidenced by the change in optical density.

Another more definitive technique to directly demonstrate the entry of malate into the mitochondria is the use of radioactively labelled compounds.  $C^{14}$  labelled malate was incubated with mitochondria in the usual manner. Tritiated water was also included in the incubation to correct for uptake due to swelling. Mitochondria were incubated with  $C^{14}$ -malate and aliquots of the suspension were removed at timed intervals. The mitochondria were quickly spun down, washed, and counted for  $C^{14}$  and  $H^3$  content. The activity of  $C^{14}$  reflected the intramitochondrial malate while the  $H^3$  counts reflected the degree of intramitochondrial water. This control was added for it was conceivable that the addition of  $P_i$  would promote mitochondrial swelling, and the increased intramitochondrial space might itself account for any rise in  $C^{14}$  activity. The determination of  $H^3$ -water reflects the water space and would correct for this swelling. The  $C^{14}$  label on the malate was so situated as to be removed as  $CO_2$  during the reaction so that  $C^{14}$  counts would not leave the mitochondria in the form of labelled pyruvate. Fig. 17 demonstrates the radioactivity of the mitochondrial pellet during the incubation. Note that upon addition of  $P_i$  there is a marked rise in pellet  $C^{14}$  counts suggesting an accumulation of intramitochondrial malate since  $H^3$ -water indicated no significant mitochondrial swelling. This response to  $P_i$  is very rapid, peaks by 2 minutes after addition, then rapidly falls off. It appears as if malate enters the mitochondria in one large influx following  $P_i$  addition. Pyruvate determination in the supernatant fluids of this experiment demonstrated a simultaneous augmentation in pyruvate efflux following  $P_i$ . This is



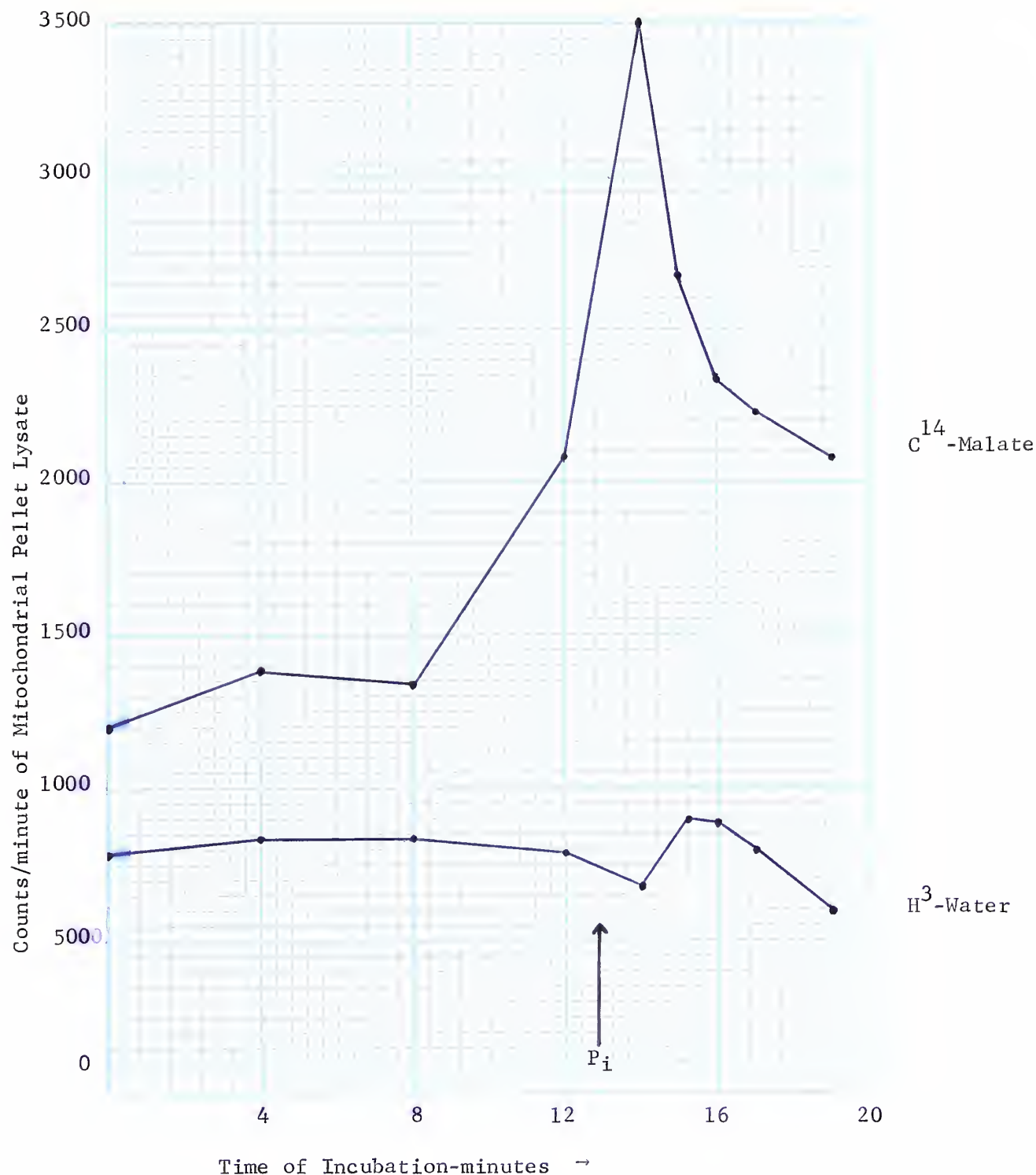


Figure 17. Effect of  $P_i$  on Mitochondrial Accumulation of  $C^{14}$ -Malate

2 flasks were incubated at  $37^\circ$  C. with the following contents: incubation medium, 10 mM malate, 2 mM KCN, 1 mM arsenite, 2 mM  $P_i$ ,  $H_2O$ , and 0.76 mgm. mitochondrial protein/ml. 1 flask contained  $i$  sufficient  $C^{14}$  labelled malate to give 317,500 cpm/ml. of incubation while the second flask contained  $H^3$ -water at 330,000 cpm/ml. of incubation. The 2 flasks were identical in all other respects.  $\frac{1}{2}$  ml. aliquots were removed at the intervals shown and the mitochondria centrifuged down in 15 sec. The surface of the pellet was washed once and the residual fluid removed. After lysing with perchloric acid, 50  $\mu$ l. of this lysate was counted for radioactivity.



consistent with malate entry. This experiment provides direct evidence for the transport of malate into the mitochondria. Since there is ample proof that pyruvate diffuses out of the KCN-inhibited mitochondria, and that this is completely dependent on malate(Fig. 13), we can be fairly certain that malic enzyme activity is responsible.

### 3. Partial Purification of Malic Enzyme and Malate Shuttle Experiments

It is well known that malate alone will support steroid hydroxylation either in the presence or absence of KCN. Since we now know that malate can enter the mitochondria and that pyruvate effluxes even in the absence of arsenite, the possibility exists that a malate shuttle might be operative in rat adrenal mitochondria. Simpson and Estabrook have reported a malate shuttle in bovine adrenal cortex mitochondria(53). Especially noteworthy is the fact that over 90% of ME activity is present in the soluble cell fraction(Fig. 8). If a malate shuttle exists in the rat, the abundant soluble ME should reform malate from pyruvate,  $\text{HCO}_3^-$ , and NADPH in the presence of  $\text{Mn}^{++}$  in amounts great enough to support steroid hydroxylation.

The supernatant ME was purified and concentrated as follows: Those fractions from the 100,000g-60 min. supernate precipitating between 65 and 87% of saturation with ammonium sulfate were dialyzed against 30 mM TRIS-HCl to remove  $\text{NH}_4^+$ . After concentrating this enzyme solution, the purification of ME activity approached 14 fold. This partially purified enzyme plus the reactants necessary for the formation of malate from pyruvate were used to reconstruct the malate shuttle as described in Table III.



After a 15 minute incubation the samples were assayed for corticosterone formation from DOC. Control flasks lacking certain of the reactants were incubated simultaneously. The results are shown in Table III.

The complete system which contained all reactants generated a 2-3 fold greater quantity of B than any of the controls (with the exception of the one lacking NADPH and  $\text{HCO}_3^-$ ).

The finding that ME from the adrenal cell soluble fraction will use pyruvate +  $\text{HCO}_3^-$  + NADPH and support steroid hydroxylation is tentative evidence that a functional malate shuttle may well exist in the rat adrenal.

TABLE III. Corticosterone Formation Supported by Extramitochondrially Generated Malate.

| FLASK                                        | nanogm. B formed/ml. incubation<br>medium |
|----------------------------------------------|-------------------------------------------|
| Complete system                              | 13,920                                    |
| w/out Pyruvate, $\text{HCO}_3^-$ , NADPH, ME | 3,860                                     |
| w/out Pyruvate, $\text{HCO}_3^-$ , NADPH     | 6,640                                     |
| w/out Pyruvate                               | 6,040                                     |
| w/out $\text{HCO}_3^-$ , NADPH               | 9,420                                     |
| w/out ME                                     | 4,040                                     |

Flasks incubated in duplicate contained the following reactants: the concentrated enzyme, 10 mM pyruvate, 50 mM  $\text{HCO}_3^-$ , 5 mM  $\text{Mg}^{++}$ , 2 mM  $\text{P}_i$ , 1 mM arsenite, 2 mM KCN, 300  $\mu\text{M}$  DOC, an NADPH generating system of 0.5 mM NADPH, 10 mM glucose-6- $\text{PO}_4$ , and glucose-6- $\text{PO}_4$  dehydrogenase, and the incubation medium. Flasks contained 0.8 mgm. mitochondrial protein/ml. A preincubation lasted 7 minutes at 37° C., and mitochondria were added at zero time to start the 15 minute incubation period. The reaction was terminated by addition of 1.0 ml. of 0.5%  $\text{HgCl}_2$  and icing. Samples were then assayed for content of B.



In order to substantiate this finding, the same experiment was repeated on two other occasions utilizing soluble ME partially purified from separate batches of pooled soluble supernatants. The experiments were altered somewhat to show the progression of B formation over time, but results to confirm the original finding were unsuccessful.

The results of the original experiment implied that malate was formed; therefore, NADPH should have been oxidized during the reaction. To confirm that this was actually occurring, the partially purified ME was incubated with NADPH in the presence of pyruvate and other factors included in the shuttle reaction. As shown in Fig. 18, NADPH oxidation was observed spectrophotometrically. This provides further evidence that ME may indeed catalyze the reverse reaction. As in the case of ICDH, the oxidation of NADPH should continue at a reasonably constant rate if there were a continual regeneration of NADP. This spectrophotometric evidence that ME can oxidize NADPH in the presence of pyruvate and  $\text{HCO}_3^-$  lends credibility to the successful demonstration of the malate shuttle experiment.

In summary, all the in vitro results obtained with ME indicate that a malate shuttle is functionally possible in rat adrenal mitochondria. Malate entry into the mitochondria is stimulated by  $\text{P}_i$  and inhibited by p-IBM. Mitochondrial ME activity can support steroid hydroxylation and result in accumulation and diffusion of pyruvate from the mitochondria. This is enhanced by the presence of arsenite and DOC. Pyruvate can be reconverted to malate by the ME of the soluble cell fraction in amounts sufficient to support mitochondrial steroid hydroxylation. A schematic representation of the possible pathways suggested by these results is shown.



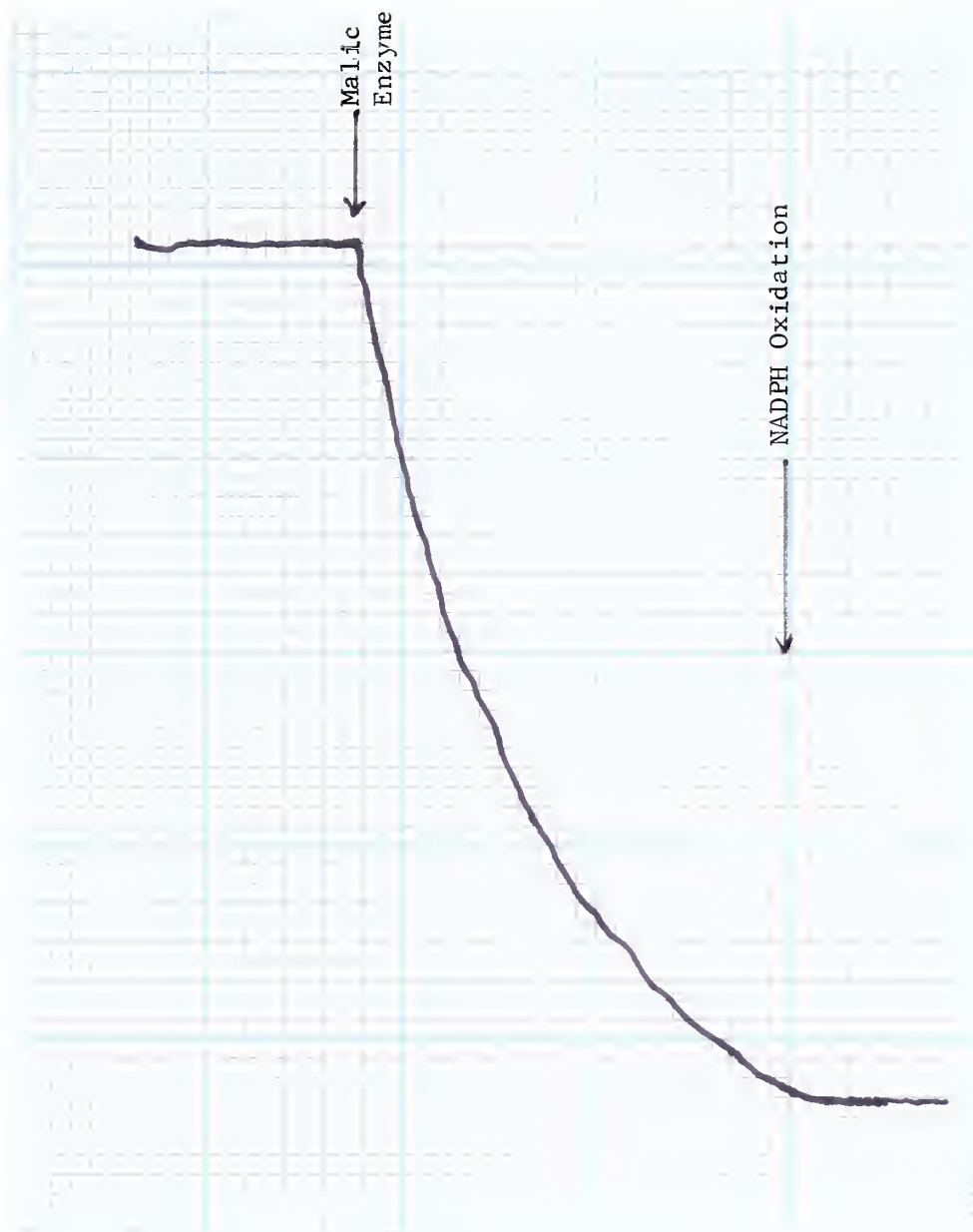
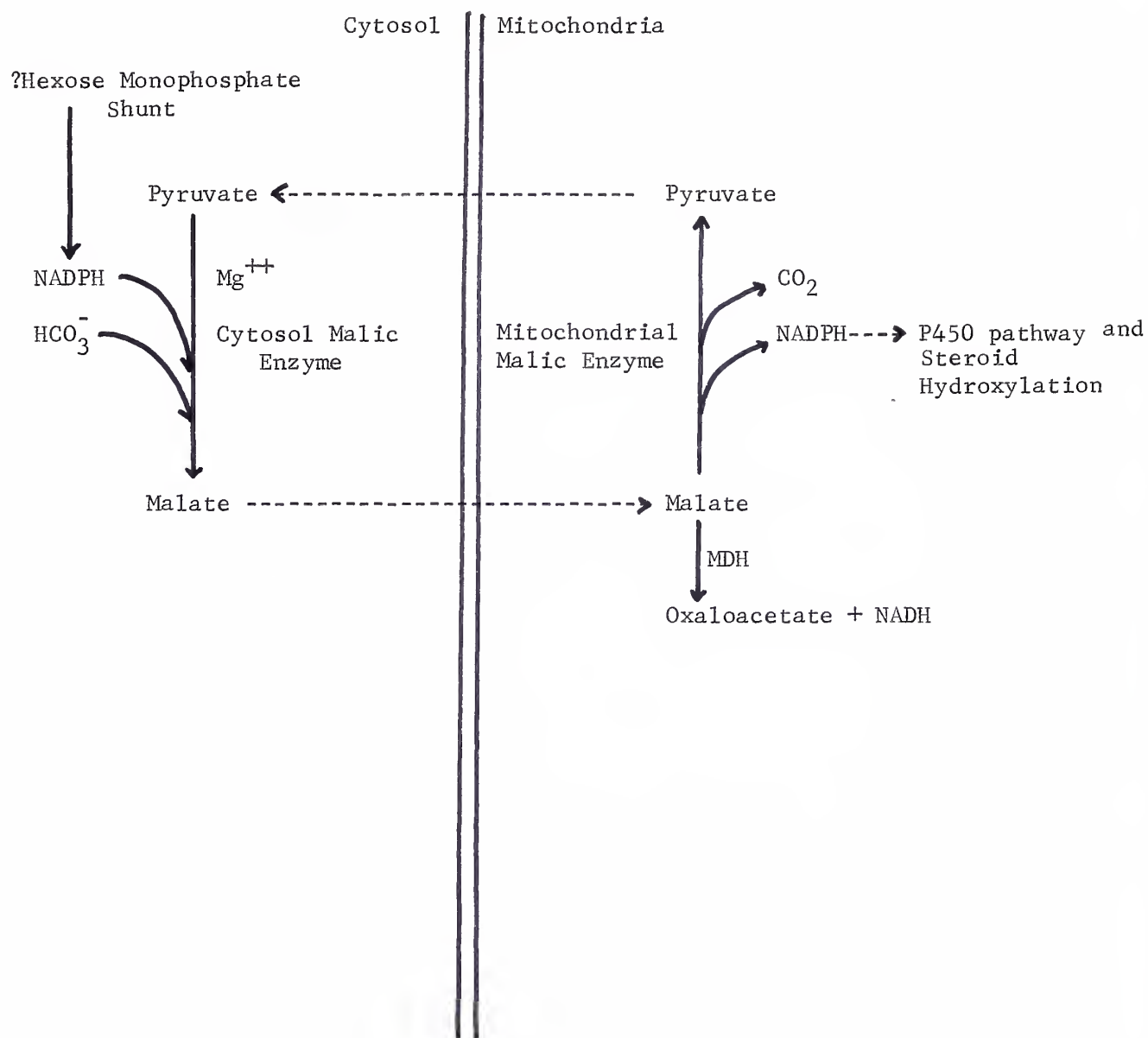


Figure 18. Spectrophotometric Oxidation of NADPH by Soluble Malic Enzyme in the Presence of Pyruvate.

Cuvette contained 4 mM pyruvate, 10 mM  $\text{Mg}^{++}$ , 50 mM  $\text{HCO}_3^-$ , NADPH, and the partially purified soluble ME. The reaction was started by the addition of ME (arrow), and oxidation of NADPH at 340 m $\mu$  occurs in the direction shown. The smallest squares on the paper represent 31.1 m $\mu$ moles of NADPH.



Figure 19. A Schematic Representation of the Malate Shuttle in the Rat Adrenal Mitochondria and Soluble Fraction





## DISCUSSION

These data have demonstrated and clarified the role of non-energy dependent mechanisms in providing intramitochondrial NADPH for rat adrenal steroid hydroxylation. The results outline certain biochemical features of isocitrate and malate metabolism in relation to steroidogenesis, and evidence is presented to support the existence of a functional malate shuttle.

NADP-linked ICDH has been shown to have an essentially bimodal distribution in various animal tissues with the soluble fraction accounting for 80-90% of the activity and the remainder being localized in the mitochondria. This is true in rat and mouse liver(35,36), bovine adrenal (39), human placenta(38), and human adrenal(24). Our data demonstrate an essentially similar distribution of the enzyme in the rat adrenal with almost 20% of the activity located in the mitochondria.

Although the distribution of NADP-linked ICDH is basically similar in these tissues, different opinions exist regarding the mitochondrial pathways of isocitrate oxidation. For example, in rat liver tissue there is evidence that either the NAD or NADP linked pathways may predominate during isocitrate oxidation(36,61). On the other hand, Grant has shown



that the NAD-linked MDH is the major pathway in bovine adrenal mitochondria (62). He provided additional evidence that isocitrate was a poor substrate for steroid 11 $\beta$  hydroxylation and therefore implied that the transhydrogenase activity was low. Our data in the rat adrenal show the NADP-linked ICDH to be highly active in the oxidation of isocitrate not only in the soluble fraction but in the mitochondria as well. The high rates of NADPH formation and  $\alpha$ -ketoglutarate efflux observed with the mitochondrial ICDH are consistent with the observations of others(11,23,34) that isocitrate, of all Krebs cycle intermediates, supports the highest rates of 11 $\beta$  hydroxylation. In addition, respiratory chain inhibitors have little or no effect on these rates suggesting that the NAD-linked pathway is of minor significance during steroid hydroxylation.

Of interest is the fact that mitochondria possess only 17% of the total NADP-linked ICDH activity. Furthermore, NAD-linked MDH is located exclusively in the mitochondria. That isocitrate seems to be preferentially oxidized by the NADP-linked ICDH during steroid hydroxylation may reflect a unique evolutionary development in the rat whereby steroidogenesis is virtually independent of oxidative phosphorylation. The advantage of such a situation may be manifest during periods of limited substrate availability which might preclude simultaneous activity of both the P450 and the classic electron transport pathways.

The purpose of a soluble NADP-linked ICDH is not fully appreciated. Goebell and Pette(39) suggested that it is related to glycolysis and serves to regulate citrate in the cytoplasm. Another possible function of the enzyme may be provision of NADPH for the microsomal NADPH-dependent



17 and 21 hydroxylations. This takes on additional significance considering recent findings from Harding's laboratory(63). These workers assayed crude rat adrenal homogenates for Krebs cycle intermediates and found isocitrate by far the most abundant substrate present. In addition to the possibilities mentioned, we feel that the presence of this highly active soluble enzyme may imply a shuttle for NADPH. Despite the fact that we were unable to demonstrate its support of steroid hydroxylation, spectrophotometric evidence shows that the shuttle is biochemically feasible. Once again, it should be emphasized that in vitro laboratory techniques can only approximate, at best, the actual intracellular milieu.

On the spectrophotometric basis of NADPH production, virtually all of the ME activity in sonicated rat adrenal tissue is localized in the soluble fraction; yet mitochondrial enzyme activity is clearly manifest on the basis of  $P_i$  stimulated pyruvate efflux during incubations with intact mitochondria. This paradoxical situation apparent with ME is not easily explained. Others have interpreted pyruvate efflux from bovine adrenal mitochondria to reflect malic enzyme activity(51,64), but no dependence on  $P_i$  was demonstrated. Using rat adrenal mitochondria, Purvis(40) and Peron(50) both provided evidence that ME was not an important pathway for malate oxidation. On the other hand, Sauer(34) found that in rat adrenal mitochondria ME could contribute about half the NADPH for steroid 11 $\beta$  hydroxylation when this pathway was stimulated by  $P_i$  (the remaining NADPH originating from MDH and the ELTH). However, unlike other mitochondrial enzymes, ME could not be demonstrated following sonication. These findings led Sauer to conclude that  $P_i$  activated a malic enzyme-like activity. Our data confirm certain of these observations: ME indeed could not be



demonstrated in the mitochondria following sonication(Fig.9). However, we were able to demonstrate ME activity in the supernatant fluid of sonicated mitochondria which had been centrifuged at 100,000g for 60 minutes(Fig. 10). This procedure eliminated the disrupted mitochondrial membrane fragments. Despite the presence of KCN in the cuvette these membranes interfered with the spectrophotometric assay for ME. Our findings therefore suggest that ME is indeed present.

The mechanism of the  $P_i$  effect has been recently explored by Chappell (59). He feels that malate, a dicarboxylic anion, can only penetrate the mitochondrial membrane by exchanging for another similarly charged anion such as  $HPO_4^{-2}$ . Since  $P_i$  easily penetrates the membrane in exchange for  $OH^-$ , the presence of adequate intramitochondrial phosphate anions permits malate entry. Why this  $P_i$  requirement for malate entry is seen in rats but not bovine mitochondria is unclear; but Sauer feels that bovine mitochondria may contain sufficient endogenous phosphate to exceed the relatively small  $K_m$  for this activation(34).

The possibility exists that the pyruvate efflux witnessed in our experiments may be a result of MDH activity rather than ME. This could occur if the mitochondria also contained an oxaloacetate decarboxylase or a NAD-linked decarboxylating malic dehydrogenase. However, these possibilities seem unlikely since  $P_i$  stimulated pyruvate efflux occurs in the presence of respiratory chain inhibitors(Fig. 11) which would tend to inhibit the NAD-linked oxidation. In addition, malate supported  $11\beta$  hydroxylation is only partly inhibited by KCN(34), suggesting that the uninhibited contribution is the NADP-linked energy independent ME.



We have provided the first evidence that a functional malate shuttle may exist in the rat adrenal for support of steroid hydroxylation. This was originally proposed by Peron, but he subsequently was unable to demonstrate its existence. The attempts to confirm our original finding were unsuccessful. Although the reasons are unclear, one possible explanation may be that the 2 mM  $P_i$  used in the experiments actually inhibited the support of steroid hydroxylation by the small amount of malate formed. Sauer has shown(65) that when malate concentrations are very low--in the range of 0.1 mM--the presence of 2 mM  $P_i$  inhibits steroid hydroxylation. It is not until higher malate concentrations are reached that  $P_i$  has a stimulating effect. Thus, unless our incubations with the concentrated soluble ME generated sufficient malate, it is unlikely that steroid hydroxylation beyond control values would have been demonstrated.

The possible existence of a malate shuttle in the rat adrenal provides an efficient mechanism whereby the translocation of substrates can mediate the transfer of extramitochondrially generated NADPH to the intramitochondrial space for steroid hydroxylation. Such a mechanism might allow the continued formation of active steroids without interfering with the oxidation of Krebs cycle intermediates via oxidative phosphorylation. The advantage of this situation would be manifest during periods of stress when both active steroid hydroxylation and oxidative phosphorylation are necessary. Energy independent reactions would allow the dissociation of the two pathways.

Harding's data referred to earlier(63) concerning Krebs cycle substrates present in rat adrenal homogenates showed that citrate-isocitrate concentrations were very high. In addition, malate was present in concentrations



8 fold higher than oxaloacetate and  $\alpha$ -ketoglutarate. Citrate-isocitrate was twice as abundant as malate. The presence of these two substrates in such high concentrations emphasizes their importance in adrenal cell function especially when viewed in light of current data concerning the roles of isocitrate and malate in steroidogenesis.

Until very recently, there was no available data regarding isocitrate and malate metabolism in relation to the effects of ACTH. Laury and McCarthy(66), however, have administered long term ACTH injections to rats and demonstrated the mitochondria to have an increased capacity to utilize isocitrate, succinate, and especially malate for the support of  $11\beta$  hydroxylation. These findings suggest that ACTH can increase the levels of specific mitochondrial enzymes, thus providing additional evidence that NADP-linked ICDH and ME may play key roles in steroid hydroxylation.

Our findings on non-energy dependent mechanisms-ICDH and ME-in the rat adrenal demonstrate some biochemical features of isocitrate and malate metabolism in relation to steroid hydroxylation. The data provide additional support to the important role which these mechanisms play in the overall scheme of steroid hydroxylation.



## SUMMARY

The following points have been established from the experimental results presented in this thesis:

1. NADP-linked ICDH and ME constitute two non-energy dependent enzymes which produce intramitochondrial NADPH for rat adrenal steroid hydroxylation.
2. ICDH and ME activities are evident in both the mitochondria and soluble cell fractions.
3.  $\alpha$ -ketoglutarate formed by ICDH can diffuse out of the mitochondria in the absence of arsenite, and this efflux is augmented by steroid hydroxylation.
4. There is spectrophotometric evidence that ICDH can catalyze the formation of isocitrate from  $\alpha$ -ketoglutarate,  $\text{HCO}_3^-$ , and NADPH, but this does not occur at rates sufficient to support steroid hydroxylation in vitro.
5. p-IBM can inhibit the  $\text{P}_i$  stimulation of malate entry into the mitochondria.
6. Mitochondrial ME activity can support steroid hydroxylation and result in accumulation and efflux of pyruvate from the mitochondria. This efflux is augmented by arsenite and DOC.
7. There is in vitro and spectrophotometric evidence that soluble fraction ME can make malate from pyruvate,  $\text{HCO}_3^-$ , and NADPH. The malate is formed in sufficient amounts to support steroid hydroxylation. This constitutes tentative evidence that the malate shuttle is operative in the rat adrenal gland.



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